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Carbon Assimilation by *Pseudomonas oxalaticus* (OXI)

4. METABOLISM OF OXALATE IN CELL-FREE EXTRACTS OF THE ORGANISM GROWN ON OXALATE*

BY J. R. QUAYLE, D. B. KEECH† AND G. A. TAYLOR
Medical Research Council Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford

(*Received* 5 *July* 1960)

The oxidation level of oxalate is such that incorporation of its carbon into cell constituents must involve a net reduction. If it is the sole carbon source for microbial growth then it must also undergo condensation reactions resulting in the synthesis of, for example, C_3 and C_4 units necessary for formation of carbohydrate and amino acid carbon skeletons (for a review of this aspect, see Kornberg, 1959). Previous work, mainly with whole cells of *Pseudomonas oxalaticus* (Quayle & Keech, 1959*a*, 1960) indicated that a reductive step precedes any condensation reactions, and that this initial reduction reaction is that of oxalyl-coenzyme A to glyoxylate. It was also suggested that successive steps involve condensation of two molecules of the glyoxylate to tartronic acid semialdehyde, which in turn is reduced to glyceric acid by a reaction sequence similar to that proposed for the growth of *Pseudomonas* species on glycollate (Kornberg & Gotto, 1959).

The formation and metabolism of oxalyl-coenzyme A, including its reduction to glyoxylate by reduced triphosphopyridine nucleotide in cell-free extracts of *P. oxalaticus*, are reported in this paper. Part of this work has been presented to the Biochemical Society (Quayle, Keech & Taylor, 1960).

METHODS AND MATERIALS

Maintenance and growth of the organism. The growth conditions have been described by Quayle & Keech (1960).

Isotopic materials. Radioactive chemicals were purchased from The Radiochemical Centre, Amersham, Bucks.

Preparation of cell-free extracts. Harvested cells (1·25–5 g.) were suspended in 4 vol. of suitable buffer, as indicated in the text, and subjected to ultrasonic vibrations for 2–3 min. with a 25 kcyc./sec. Mullard ultrasonic generator type E 7590 B (Mullard Ltd., London); to minimize heating, the transducer assembly was immersed in a rapidly flowing stream of tap water. The resulting suspension was centrifuged at 25 000 *g* for 20 min. in a refrigerated angle-head centrifuge; this crude supernatant fraction which contained 10–30 mg. of protein/ml. was used as such in many experiments. For some purposes the crude extract was either dialysed for 3 hr. against 2 l. of 10 mM-buffer at 3° before use, or the protein was precipitated at 0° and pH 7·0 by the addition of saturated $(NH_4)_2SO_4$ solution to a final strength of 80% of saturation. The resulting precipitated protein was collected by centrifuging at 0°, dissolved in 5–10 ml. of 10 mM-buffer and dialysed for 3 hr. against 2 l. of the same buffer at 3°.

In one experiment (Table 8) a bacterial extract was prepared by crushing a frozen suspension of the bacteria, containing 1 g. wet wt. suspended in 2 ml. of 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer (0·01 M, pH 7·3) in a Hughes (1951) press at −20°. The crushed material was homogenized and about 200 μg. each of crystalline ribonuclease and deoxyribonuclease (L. Light and Co. Ltd., Colnbrook, Bucks.) were added.

Manometric methods. The decarboxylation and oxidation of oxalate was measured in conventional Warburg manometric apparatus.

Radioactive assay of $^{14}CO_2$, [^{14}C]*oxalate and* [^{14}C]*formate.* $^{14}CO_2$ was converted into $Ba^{14}CO_3$ by precipitation with $BaCl_2$ and was then plated on paper disks (2·4 cm. diam.) as described by Sakami (1955*a*). The samples were assayed for radioactivity in a gas-flow counter (Model D-47, Nuclear Instrument and Chemical Corp., Chicago, U.S.A.), and corrected for self-absorption. Study of the enzymic decarboxylation of oxalic acid to formic acid involved the use of [^{14}C]oxalic acid of known specific activity in terms of $Ba^{14}CO_3$ derived from it by oxidation. The specific activity of the [^{14}C]oxalate was determined by oxidation to $^{14}CO_2$ with $KMnO_4$ (0·4 N, in 2 N-H_2SO_4) at 60–80° and the evolved $^{14}CO_2$ was trapped in CO_2-free NaOH solution as described by Sakami (1955*b*). The resulting $Na_2{}^{14}CO_3$ was converted into $Ba^{14}CO_3$ and assayed as above. The amount of [^{14}C]-formate evolved from [^{14}C]oxalic acid by the cell-free extracts was estimated by adding 1·96 m-moles of unlabelled formic acid to the acidified reaction mixture at the end of the reaction. The formic acid was steam-distilled out of the mixture and was then decarboxylated by Mg^{2+} ions in acetate buffer as described by Sakami (1955*c*); the yields of CO_2 obtained were 70–80% of theoretical. The specific activity of the evolved $^{14}CO_2$ was measured as $Ba^{14}CO_3$, and from this value the yield of [^{14}C]formate from the [^{14}C]oxalate in the original reaction mixture was calculated.

Chromatographic and radioautographic analysis. The general techniques used for chromatography and radioautography of radioactive compounds have been described by Quayle & Keech (1959*b*). Formate was chromatographed in ethanol–aq. NH_3 soln. (sp.gr. 0·880)–water

* Part 3: Quayle & Keech (1960).

† Present address: Department of Biochemistry, Western Reserve University, Cleveland, Ohio, U.S.A.

Table 1. *Decarboxylation of oxalate by the crude bacterial extract*

The main compartment of each Warburg vessel contained 50 μmoles of sodium potassium phosphate buffer (pH 6·9) in Expt. 1, or 50 μmoles of tris buffer (pH 6·9) in Expt. 2, and ATP and oxalate as indicated; the two side arms contained respectively 0·5 ml. of cell-free extract, and 0·25 ml. of 10 N-H_2SO_4 in Expt. 1 or 0·2 ml. of aq. 50% trichloroacetic acid (w/v) in Expt. 2. Total vol. of contents, 2 ml. in Expt. 1 or 2·2 ml. in Expt. 2. The flasks were flushed with N_2 and incubated at 30° for 30 min. in Expt. 1, or 15 min. in Expt. 2, before the acid was tipped in from the second side arms. In Expt. 2 the flask contents were centrifuged, the solid residues extracted with 1 ml. of water, and the combined supernatants were made up to 4 ml. and neutralized with 0·25 ml. of M-Na_2CO_3. Samples (0·5 ml.) were analysed by ion-exchange chromatography on columns of Dowex-1 (Cl^- form). The extracts were prepared from 1 g. of cells subjected to ultrasonic vibrations for 4 min. in 3 ml. of 0·1 M-phosphate buffer (pH 6·9) in Expt. 1, or 5 ml. of 0·01 M-tris buffer (pH 6·9) in Expt. 2, followed by centrifuging.

Expt. no.	Amount of oxalate in incubation mixture (μmoles)	Amount of ATP in incubation mixture (μmoles)	CO_2 evolved (μmoles)	Amount of adenine nucleotides present after incubation (μmoles)		
				AMP	ADP	ATP
1	20	0	0·8	—	—	—
	20	1	3·2	—	—	—
	20	5	10·6	—	—	—
	20	10	16·4	—	—	—
	20	20	19·4	—	—	—
Analysis of original ATP solution used	—	—	—	0	0	22·0
2	20	22	13·1	2·5	5·2	14·3
	0	22	0·31	2·5	5·2	15·0

(80:4:16, by vol.; Long, Quayle & Stedman, 1951). Succinyl-, formyl- and oxalyl-coenzyme A were chromatographed in phenol–water (1:1, v/v; Fink & Fink, 1949; Jakoby, Ohmura & Hayaishi, 1956) and located by spraying with 5% (w/v) $FeCl_3,6H_2O$ in ethanol (0·1 N in HCl) (Stadtman & Barker, 1950).

Preparation of succinyl-coenzyme A. The method of Simon & Shemin (1953) was used with slight modification. Coenzyme A (CoA, 15 mg., Pabst Laboratories, Milwaukee, Wis., U.S.A.) was dissolved in 2 ml. of water at 0°, and 0·25 ml. of aqueous 0·1 M-succinic anhydride was added; the pH was adjusted to 7 with 0·1 N-NaOH, and the solution kept at 0° for 20 min. At the end of this time a sample gave a negative nitroprusside test for free thiol groups; this became positive after a sample was first treated with methanolic KOH, indicating that the formation of the succinyl-CoA was complete (Stadtman, 1957). The succinyl-CoA content of the solution, determined by the hydroxamic acid method of Lipmann & Tuttle (1945), showed yields of acyl-CoA of 30–50% of the theoretical yield expected from the weight of CoA taken. It was used immediately after preparation, owing to its instability.

Protein determinations. Protein was estimated by the method of Folin & Ciocalteu (1927).

Analysis of adenine nucleotides. Mixtures of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) were separated by standard procedures of ion-exchange chromatography. Approx. 3 μmoles of nucleotide, contained in a 0·5 ml. solution, was adsorbed on a column (4 cm. long × 1·5 cm. diameter) of Dowex-1 (Cl^- form, 200–400 mesh). The AMP, ADP and ATP were successively eluted from the column by washing with 10-ml. portions of 0·01 N-HCl, 0·01 N-HCl–0·05 M-NaCl and 0·01 N-HCl–0·2 M-NaCl respectively. Their quantities were assayed spectrophotometrically at 260 mμ.

Chemicals. A sample of pure sodium glyoxylate was a gift from Dr I. Zelitch.

Table 2. *Stoicheiometry of the decarboxylation of oxalate*

The complete system in the main compartments of the Warburg vessels contained 50 μmoles of tris–HCl buffer (pH 7·0), 20 μmoles of ATP, 20 μmoles of [^{14}C]oxalate (0·125 μc of ^{14}C) of known specific activity in terms of derived $Ba^{14}CO_3$; the two side arms contained respectively 0·5 ml. of cell-free extract (or boiled extract) and 100 μmoles of H_2SO_4 (0·2 ml.). Total volume of contents was 2·2 ml. The flasks were flushed with N_2 and incubated at 30° for 60 min. before the acid was tipped in. The extract was prepared from 1·25 g. of cells subjected to ultrasonic vibrations in 5 ml. of 10 mM-tris–HCl buffer (pH 7·0) for 3 min. and centrifuged. The contents of the flasks were analysed for [^{14}C]formate by isotope dilution. In this measurement, 1·96 m-moles of formic acid was added to the flask contents; the formic acid was then isolated, purified, and decarboxylated to $^{14}CO_2$. The $^{14}CO_2$ was converted into $Ba^{14}CO_3$, the specific activity of which was measured.

Omissions	Reaction products	
	CO_2 (μmoles)	Formate (μmoles)
None	15·2	14·1
ATP	5·1	3·5
Boiled extract used in place of enzyme extract	0	0

RESULTS

Decarboxylation of oxalate

The crude supernatant fraction from cell-free extracts of *P. oxalaticus* prepared by ultrasonic disintegration catalysed a rapid decarboxylation of oxalate in the presence of ATP (Table 1). ATP

was required in catalytic quantities and up to 1 mole of carbon dioxide was evolved from 1 mole of oxalate. Other experiments have shown that no more than 1 mole of carbon dioxide could be evolved from 1 mole of oxalate even in the presence of more than 1 mole of ATP. The requirement for ATP could be met with inosine triphosphate, uridine triphosphate or cytidine triphosphate.

Products and stoicheiometry of the decarboxylation. With [^{14}C]oxalate as substrate in the above reaction mixture, the major product was identified chromatographically as [^{14}C]formate. The stoicheiometry of the reaction was determined by using [^{14}C]oxalate of known specific activity and estimating the resulting [^{14}C]formate by the method of isotope dilution (Table 2). The evolution of 15·2 μmoles of CO_2 was also accompanied by the formation of 14·1 μmoles of formate; these yields were decreased to one-third when ATP was left out of the reaction mixture. That the reaction is an enzymic one is shown by the complete loss of activity after boiling the enzyme extract for 5 min.

Cofactor requirements. Jakoby *et al.* (1956) described a partially purified enzyme preparation, obtained from an organism grown in the presence of oxalate, which catalysed an anaerobic decarboxylation of oxalate. The system required substrate quantities of ATP and catalytic quantities of acetate, CoA, thiamine pyrophosphate (TPP) and magnesium ions. These authors suggested that the decarboxylation proceeded by the following reactions:

$$\text{Acetyl-CoA} + \text{oxalate} \rightleftharpoons \text{oxalyl-CoA} + \text{acetate} \quad (1)$$

$$\text{Oxalyl-CoA} \rightarrow [\text{formyl-CoA}] + CO_2 \quad (2)$$

$$[\text{Formyl-CoA}] + H_2O \rightarrow \text{formate} + \text{CoA} \quad (3)$$

The function of the ATP, acetate and CoA in the overall reaction was to synthesize acetyl-CoA by the action of acetyl-CoA synthetase (acetic thiokinase). The enzyme preparation used was prepared from bacterial extracts, which had been subjected to ultrasonic vibrations, by precipitation with ammonium sulphate between 25 and 50% saturation.

An enzyme fraction was prepared in a similar fashion from oxalate-grown *P. oxalaticus* and was tested for decarboxylation activity manometrically. However, the carbon dioxide output was too small to be measured in this way, hence [^{14}C]-oxalate was used as substrate and the evolved $^{14}CO_2$ was collected in the centre wells of the Warburg vessels and assayed as barium [^{14}C]-carbonate.

The decarboxylation was dependent on the presence of acetate, ATP and CoA (Table 3); omission of any of these cofactors caused a six- to eight-fold diminution of activity. This closely followed the findings of Jakoby *et al.* (1956). Considerable variation of the overall reaction rate attained with the complete system was encountered from one enzyme preparation to another; in no case, however, did the rate exceed 2 μmoles of carbon dioxide evolved/30 min. The same results were found when the protein fraction precipitating between 0 and 80% of ammonium sulphate saturation was used. These rates, which are of the same order as those reported by Jakoby *et al.* (1956), are nevertheless a small fraction of the decarboxylation rates which crude extracts were capable of catalysing in the presence of ATP, which suggested that some essential cofactor had been lost on purification. This was confirmed by the finding that oxalate decarboxylation by the 0–80% ammonium sulphate fraction was greatly stimulated by the addition of either boiled extract or yeast extract (Difco Laboratories, Detroit, Mich., U.S.A.). Since the yeast extract contained succinate, the effect of addition of this compound to the oxalate-decarboxylation system was tested.

Table 3. *Cofactors for the decarboxylation of oxalate*

The complete system in the main compartments of the Warburg vessels contained 100 μmoles of sodium potassium phosphate buffer (pH 6·9), 100 μmoles of NaF, 10 μmoles of $MgCl_2$, 10 μmoles of GSH, 20 μmoles of sodium acetate, 10 μmoles of ATP, 0·25 μmole of CoA, 0·1 μmole of TPP, 2·1 μmoles of [^{14}C]oxalate (1·25 μc of ^{14}C) of known specific activity in terms of derived $Ba^{14}CO_3$; the two side arms contained respectively 0·25 ml. of enzyme extract, and 2·5 m-moles of H_2SO_4 (0·25 ml.); the centre wells contained 0·2 ml. of CO_2-free 2N-KOH. The total volume was 2·75 ml. The flasks were flushed with N_2 and incubated at 30° for 30 min. before the acid was tipped in. They were then shaken for a further 30 min. before the contents of the centre wells were analysed by adding 115 μmoles of Na_2CO_3 to each and measuring the specific activity of the [^{14}C]-carbonate as $Ba^{14}CO_3$. The enzyme fraction was prepared by precipitating the protein from a sonic extract of 4 g. wet wt. of bacteria with $(NH_4)_2SO_4$ between 25 and 50% saturation. The precipitated protein was dissolved in 1 ml. of 50 mM-sodium potassium phosphate buffer (pH 6·9) and dialysed for 4 hr. against 4 l. of the same buffer containing 20 m-moles of GSH.

Omissions	CO_2 evolved (μmoles)
None	0·031
Sodium acetate	0·0059
ATP	0·0039
CoA	0·0062
TPP	0·031

Succinate requirement. Oxalate was rapidly decarboxylated by the 0–80% ammonium sulphate fraction, in the presence of ATP, CoA, TPP, magnesium chloride, reduced glutathione (GSH) and catalytic quantities of succinate (Fig. 1). In

the absence of succinate, CoA or oxalate no decarboxylation was detected manometrically. Varied requirements for the other cofactors were found. The results indicated that oxalate was decarboxylated by the scheme of reactions (1)–(3) except that acetyl-CoA was replaced in (1) by succinyl-CoA. If this were so, the function of the ATP, CoA and succinate would be to generate succinyl-CoA, and therefore the presence of the latter compound should replace the former three cofactors in the decarboxylation. This was found to be the case (Table 4): the presence of 5·7 μmoles of succinyl-CoA caused the decarboxylation of 9·5 μmoles of oxalate. The non-stoicheiometric relationship between succinyl-CoA and oxalate decarboxylation indicated that oxalyl-CoA must be regenerated after the decarboxylation step by transfer of the CoA grouping from formyl-CoA, either directly to oxalate, or indirectly via succinate.

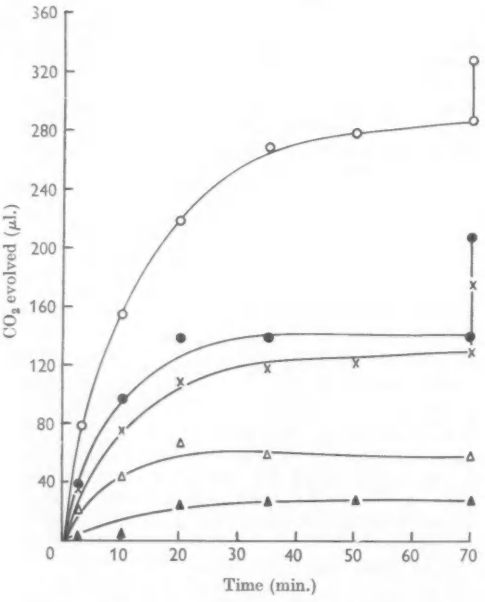


Fig. 1. Effect of succinate and other cofactors on the anaerobic decarboxylation of oxalate. The complete system in the main compartment of the Warburg vessels contained 50 μmoles of tris buffer (pH 7·2), 20 μmoles of GSH, 10 μmoles of ATP, 0·5 μmole of CoA, 0·5 μmole of TPP, 10 μmoles of $MgCl_2$, 1 μmole of sodium succinate and 20 μmoles of potassium oxalate; the two side arms contained respectively 0·35 ml. of enzyme extract, and 0·25 ml. of aq. trichloroacetic acid (50%, w/v). The total volume was 2·25 ml. The flasks were flushed with N_2 and incubated at 30°. The reaction was stopped and bound CO_2 liberated by tipping in the acid after 70 min. incubation. The enzyme extract was a 0–80% $(NH_4)_2SO_4$ fraction. There was no measurable CO_2 output on omitting oxalate, succinate or CoA singly from the incubation mixture. ○, Complete system; ●, $MgCl_2$ omitted; ×, GSH omitted; △, TPP omitted; ▲, ATP omitted.

Formation of succinyl-coenzyme A from succinate. The preceding results indicate that a succinyl-CoA synthetase is present in *P. oxalaticus* which synthesizes succinyl-CoA from succinate, ATP and CoA. The presence of such an enzyme was demonstrated by the method described by Jones & Lipmann (1955) for acetyl-CoA synthetase; succinate was used in place of acetate, and the buffer used was tris buffer (pH 7·6). In this assay, the succinyl-CoA is detected as its derived hydroxamic acid with a ferric chloride reagent. With either the crude, cell-free extract or the 0–80% ammonium sulphate fraction, rates of succinylhydroxamate formation of 0·8 μmole/hr./mg. of protein were observed. Negligible hydroxamate formation was observed if acetate, oxalate or formate was substituted for succinate in the assay system. Succinylhydroxamate formed in the complete incubation system was identified chromatographically.

Formation of oxalylhydroxamate and formylhydroxamate. The presence of oxalyl-CoA and formyl-CoA in the incubation mixture during the decarboxylation of [^{14}C]oxalate was demonstrated by adding hydroxylamine to the mixture and chromatographing the products. After incubation of 4·6 μmoles of succinyl-CoA with 9 μmoles of [^{14}C]oxalate, radioactive formylhydroxamate and oxalylhydroxamate, and non-radioactive succinylhydroxamate, were detected on the chromatogram (Table 5). The amounts of formylhydroxamate and oxalylhydroxamate isolated were very small: under the conditions of radioassay their radioactivity

Table 4. *Replacement of the requirement for succinate, adenosine triphosphate and coenzyme A by succinyl-coenzyme A in the decarboxylation of oxalate*

The main compartment of each Warburg vessel contained 50 μmoles of tris buffer (pH 7·2), 20 μmoles of GSH, 0·5 μmole of TPP, 10 μmoles of $MgCl_2$, 20 μmoles of potassium oxalate and additions as indicated; the two side arms contained respectively 0·5 ml. of enzyme extract [0–80% $(NH_4)_2SO_4$ fraction], and 0·25 ml. of aq. trichloroacetic acid (50%, w/v). The total volume was 3·6 ml. The flasks were flushed with N_2, and incubated at 30° for 40 min. before the acid was tipped in.

Additions	CO_2 evolved (μmoles)
None	0
ATP (10 μmoles) + CoA (0·5 μmole) + succinate (1 μmole)	17
Succinyl-CoA (5·7 μmoles)	9·5

Table 5. *Formation of oxalylhydroxamate and formylhydroxamate during the anaerobic decarboxylation of oxalate*

The main compartment of each Warburg vessel contained 50 μmoles of tris buffer (pH 7·2), 20 μmoles of GSH, 0·5 μmole of TPP, 10 μmoles of $MgCl_2$, 9 μmoles of [^{14}C]oxalate (0·83 μc of ^{14}C) and succinyl-CoA as indicated; the two side arms contained respectively 0·35 ml. of enzyme extract [0–80% $(NH_4)_2SO_4$ fraction], and 1 m-mole of neutral hydroxylamine (0·5 ml.). The total vol. was 3·65 ml. The flasks were flushed with N_2 and incubated at 30°. After incubation for 8 min. the hydroxylamine was tipped in, the flask shaken for 10 min., and the contents were then tipped into 30 ml. of ethanol and centrifuged. The supernatant was evaporated to dryness under reduced pressure of N_2, the residue was extracted with 3 ml. of ethanol, concentrated and chromatographed in phenol–water (1:1, w/v). The paper was dried and sprayed with $FeCl_3$ reagent.

Addition	CO_2 evolved (μmoles)	Hydroxamates detected	R_F	Radioactivity detected in hydroxamates (counts/min.)
Succinyl-CoA (4·6 μmoles)	3	Succinyl	0·46	0
		Oxalyl	0·08	32
		Formyl	0·54	270
None	0·7	None	—	—

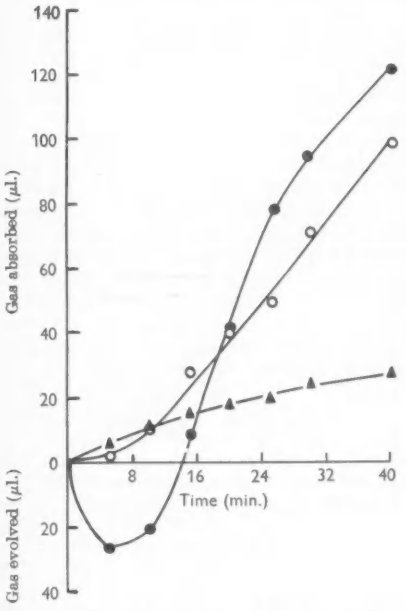


Fig. 2. Oxidation of oxalate and formate by washed suspensions of *P. oxalaticus*. The main compartments of Warburg vessels contained: 50 μmoles of potassium phosphate buffer (pH 7·0), bacteria (2 mg. dry wt.) washed with 0·9% KCl; the centre wells contained 0·2 ml. of 3 N-KOH; the side arms contained 20 μmoles of sodium formate or sodium oxalate. The final volume was 2·2 ml. The flasks were incubated at 30°. ○, Formate; ●, oxalate; ▲, endogenous.

corresponded to the presence of 0·17 and 0·01 μmole respectively. Authentic formylhydroxamate and oxalylhydroxamate were prepared chemically from the monoethyl esters of the parent acids by the method of Hestrin (1949), and succinylhydroxamate was prepared from succinic anhydride as described by Lipmann & Tuttle (1945).

Oxidation of oxalate and formate

Whole cells of *P. oxalaticus* oxidized both oxalate and formate (Fig. 2), the latter substrate after a variable lag of approx. 10 min. With oxalate, there was an initial evolution of carbon dioxide, which was more rapid than its absorption by the alkali in the centre well of the Warburg vessel. This finding suggests that the oxidation of oxalate in whole cells is preceded by decarboxylation to formate.

The organism possesses a diphosphopyridine nucleotide (DPN)-linked formic dehydrogenase; cell-free extracts catalysed the reduction of 5·3 μmoles of DPN/hr./mg. of bacterial protein when assayed according to the procedure of Nason & Little (1955). No reduction of triphosphopyridine nucleotide (TPN) was observed under these conditions.

No evidence was found for the existence of a direct DPN- or TPN-linked oxalic acid dehydrogenase in the cell-free extracts of the organism.

Interconversion of oxalate and glyoxylate

It has been suggested (Quayle & Keech, 1959*a*) that the primary step in the conversion of oxalate into cell constituents in *P. oxalaticus* is a reduction

of oxalate to glyoxylate, possibly by a CoA- and pyridine nucleotide-dependent reaction:

$$\text{Oxalyl-CoA} + \text{DPNH (or TPNH)} + \text{H}^+ \rightarrow \text{glyoxylate} + \text{DPN}^+ \text{ (or TPN)}^+ + \text{CoA} \quad (4)$$

This reaction would be analogous to the reduction of acetyl-CoA to acetaldehyde observed in *Clostridium kluyveri* by Burton & Stadtman (1953):

$$\text{Acetyl-CoA} + \text{DPNH} + \text{H}^+ \rightleftharpoons \text{acetaldehyde} + \text{DPN}^+ + \text{CoA} \quad (5)$$

Extracts of *P. oxalaticus* catalysed the reverse reaction, i.e. the reduction of TPN in the presence of glyoxylate and CoA (Fig. 3). The reaction was specific for TPN, no detectable reduction of DPN in place of TPN being observed. The rate of TPN reduction markedly increased with increasing pH, the maximum rate being observed at pH 8·5 (Fig. 4). The reaction was inhibited when tris buffer was used in place of pyrophosphate buffer at the same molarity and pH: both the initial rate and the final amount of TPN reduction were approximately halved. The partially purified enzyme (50–80 % ammonium sulphate fraction) was not very stable stored at 0° in 10 mM-sodium pyrophosphate buffer (pH 7·0). Such preparations lost up to 80 % of their activity at 0° in 24 hr.; they were, however, stable when stored at −15°. In crude, cell-free extracts the enzyme was even less stable, losing 70 % of its activity in 6 hr. at 0°.

Reaction products and stoicheiometry. The only reaction product isolated from the action of the TPN-linked glyoxylic dehydrogenase was oxalic acid. This was separated from residual glyoxylate by precipitation of the oxalate as its calcium salt. Such a procedure was used, owing to the unsatisfactory direct chromatographic and electrophoretic separations of these two acids. The precipitation method, however, precluded the use of pyrophosphate as buffer in the incubation mixture, owing to its precipitation as a gel on the addition of calcium ions. Tris buffer did not interfere in this manner and was thus used in place of pyrophosphate, although the reaction gave lower yields of product as a result. With [^{14}C]glyoxylate as substrate, the incubation was carried out in quartz cells and the reduction of TPN followed spectrophotometrically. The reaction was stopped by heating the reaction mixtures to boiling and the amount of [^{14}C]oxalate isolated from them was estimated by radioactive assay (Table 6). The identity of the product was

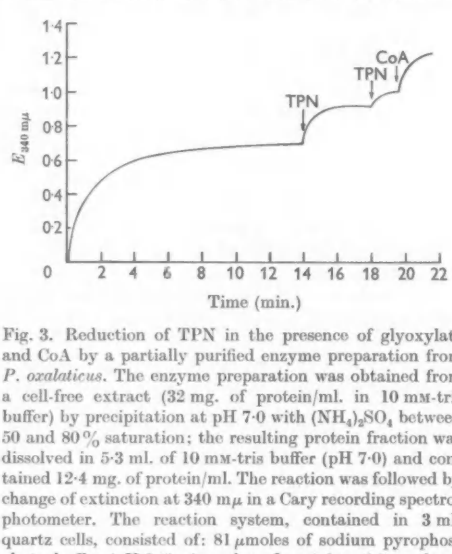


Fig. 3. Reduction of TPN in the presence of glyoxylate and CoA by a partially purified enzyme preparation from *P. oxalaticus*. The enzyme preparation was obtained from a cell-free extract (32 mg. of protein/ml. in 10 mM-tris buffer) by precipitation at pH 7·0 with $(NH_4)_2SO_4$ between 50 and 80 % saturation; the resulting protein fraction was dissolved in 5·3 ml. of 10 mM-tris buffer (pH 7·0) and contained 12·4 mg. of protein/ml. The reaction was followed by change of extinction at 340 mμ in a Cary recording spectrophotometer. The reaction system, contained in 3 ml. quartz cells, consisted of: 81 μmoles of sodium pyrophosphate buffer (pH 8·5), 4 μmoles of cysteine, 0·5 μmole of CoA, 0·5 μmole of TPN (omitted from blank cell), 10 μmoles of sodium glyoxylate, 0·1 ml. of enzyme extract and water to 3·0 ml. The reaction was started by addition of the glyoxylate. Further additions of 0·5 μmole of TPN and CoA were made as indicated.

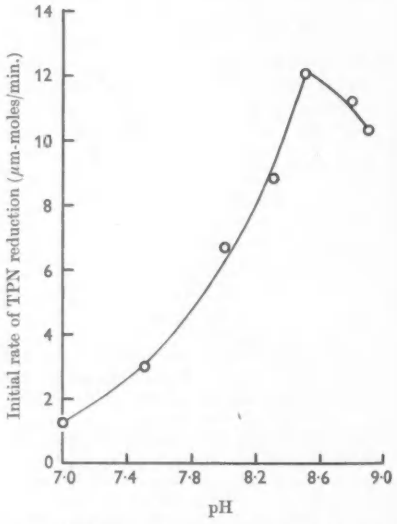


Fig. 4. pH optimum of glyoxylic dehydrogenase. The reaction was followed by measurement of TPN reduction in a Cary recording spectrophotometer at 340 mμ. The reaction system, contained in 3 ml. quartz cells, consisted of: 81 μmoles of sodium pyrophosphate buffer, 4 μmoles of cysteine, 0·5 μmole of CoA, 0·5 μmole of TPN (omitted from blank cell), 10 μmoles of sodium glyoxylate, 0·05 ml. of enzyme extract [0–80 % $(NH_4)_2SO_4$ fraction, diluted 1:10] and water to 3·0 ml. The reaction was started by addition of the glyoxylate.

Table 6. *Oxidation of glyoxylate to oxalate in the presence of coenzyme A and triphosphopyridine nucleotide*

The complete system contained: 75 μmoles of tris buffer (pH 8·5), 4 μmoles of cysteine, 1 μmole of CoA, 1 μmole of TPN, 0·22 μmole of sodium [1-^{14}C]glyoxylate ($4{\cdot}4 \times 10^4$ counts/min. of ^{14}C), 0·2 ml. of partially-purified enzyme extract [50–80 % $(NH_4)_2SO_4$ fraction containing 12·4 mg. of protein/ml.], omissions as indicated and water to 3 ml. After incubation at room temperature for 6 min., the reaction was terminated by boiling for 5 min. after 2 μmoles of carrier potassium oxalate had been added to each tube. Protein was centrifuged down and 0·05 ml. of 0·1 M-$CaCl_2$ added to each supernatant. The calcium oxalate was removed by centrifuging, washed with 0·5 ml. of water, and converted into oxalic acid by treatment with 0·1 mg. of Dowex-1 (H^+ form) and 0·1 ml. of 0·2 M-ethylenediaminetetra-acetic acid in a final vol. of 0·6 ml. After centrifuging, the resin was extracted with two 0·5 ml. portions of water which were combined with the supernatant solution. Samples (0·1 ml.) of the resulting solutions were plated on glass planchets and assayed for radioactivity.

Omissions	[^{14}C]Oxalate formed (counts/min. in total sample)	Net amount of [^{14}C]oxalate produced* (μmole)	Net amount of TPN reduced* (μmole)
None	12 900	0·054	0·048
TPN	2 060	—	—
CoA	[360]†	—	—
Boiled extract used in place of enzyme extract	[525]†	—	—

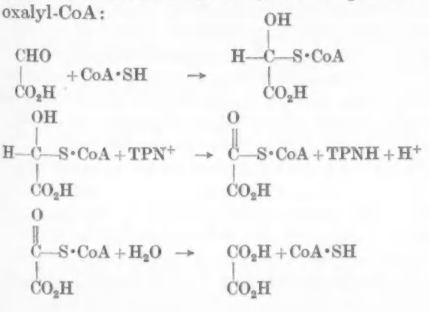
* Corrected for the value obtained with TPN omitted.
† Radioactivity not affected by oxalic acid decarboxylase treatment, probably [1-^{14}C]glyoxylate cross-contamination.

established by showing its decarboxylation to [^{14}C]formic acid on incubation with purified oxalic acid decarboxylase obtained from *Collybia velutipes* according to Shimazono & Hayaishi (1957) (Table 7).

The formation of 0·054 μmole of oxalic acid was accompanied by the reduction of 0·048 μmole of TPN (Table 6). Separate experiments in which excess of TPN and CoA were used in the presence of limiting glyoxylate, with pyrophosphate as buffer, showed that under these conditions the TPN reduction proceeds to within 90–95 % of the amount of glyoxylate added. No products of the reaction, volatile or non-volatile, other than [^{14}C]oxalate have been detected chromatographically. The results show that the reaction is represented by:

$$\text{Glyoxylate} + TPN^+ + H_2O \xrightarrow{\text{CoA}} \text{oxalate} + TPNH + H^+ \qquad (6)$$

Role of coenzyme A. It seems likely that reaction (6), by analogy with the oxidation of acetaldehyde (Burton & Stadtman, 1953), would proceed via oxalyl-CoA:



$$\text{CHO}(\text{CO}_2\text{H}) + \text{CoA·SH} \rightarrow \text{H—C(OH)(CO}_2\text{H)—S·CoA} \qquad (7)$$

$$\text{H—C(OH)(CO}_2\text{H)—S·CoA} + TPN^+ \rightarrow \text{C(=O)(CO}_2\text{H)—S·CoA} + TPNH + H^+ \qquad (8)$$

$$\text{C(=O)(CO}_2\text{H)—S·CoA} + H_2O \rightarrow \text{CO}_2\text{H—CO}_2\text{H} + \text{CoA·SH} \qquad (9)$$

The product isolated from the oxidation of glyoxylate in the previous experiment was oxalate and all attempts to detect as oxalylhydroxamate any oxalyl-CoA formed in the reaction have been unsuccessful. This may be due to its enzymic or chemical hydrolysis as in (9) since only catalytic quantities of CoA are required for the oxidation. This effect is shown in Fig. 5 where 0·015 μmole of CoA (according to the stated purity of the CoA) resulted in the reduction of 0·069 μmole of TPN in 19 min.; the reaction was still proceeding at the end of this time.

Reversibility of the reaction. In the absence of substrate quantities of oxalyl-CoA, a direct demonstration of the reversibility of the reaction is not possible. However, oxidation of TPNH, dependent on the presence of ATP, CoA, succinate and oxalate, was catalysed by a 0–80 % ammonium sulphate fraction of the cell-free extract (Fig. 6). No TPNH oxidation was observed if oxalate or any of the cofactors was omitted, suggesting that the oxalyl-CoA known to be generated in such an enzyme system from oxalate, ATP, CoA and succinate was being reduced by TPNH. Only trace amounts of glyoxylate could be detected as its 2:4-dinitrophenylhydrazone in the reaction mixture. This is to be expected in view of the presence in the enzyme extract of a highly active enzyme, similar to that discovered in *Escherichia coli* by Krakow & Barkulis (1956), catalysing the condensation

Table 7. *Identification of oxalate as the product of oxidation of glyoxylate*

A portion (1 ml.) of each of the supernatant solutions described in Table 6 was added to 0·1 ml. of 0·5 M-potassium citrate buffer (pH 4·5). Samples (0·25 ml.), adjusted to pH 7·0, were withdrawn for chromatography in ammoniacal aq. ethanol. To the remainder was added 0·1 ml. of oxalic acid decarboxylase and after 1½ hr. incubation 0·1 ml. samples were withdrawn for chromatography. The [^{14}C]formate was located by radioautography, and co-chromatographed with authentic formate. The radioactive areas on the paper chromatograms were assayed directly for radioactivity.

	Before incubation with oxalic acid decarboxylase		After incubation with oxalic acid decarboxylase	
	Radioactivity (counts/min.)		Radioactivity (counts/min.)	
Omissions (see Table 6)	Origin position of chromatogram	Formate area of chromatogram (R_F 0·54)	Origin position of chromatogram	Formate area of chromatogram (R_F 0·54)
None	535	0	0	263
TPN	96	0	0	69
Boiled extract used	10	0	10	0

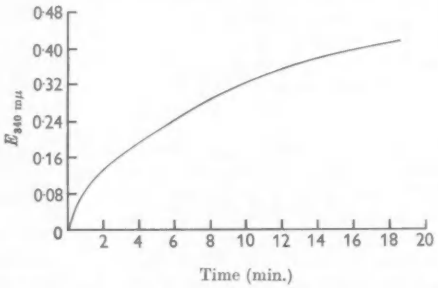


Fig. 5. Effect of a limiting amount of CoA on the oxidation of glyoxylate by TPN. The reaction was followed by change of extinction at 340 mμ in a Cary recording spectrophotometer. The reaction system, contained in 1 ml. quartz cells, consisted of: 24 μmoles of sodium pyrophosphate buffer (pH 8·5), 2 μmoles of cysteine, 0·015 μmole of CoA, 1 μmole of TPN, 0·1 ml. of partially purified enzyme [50–60% $(NH_4)_2SO_4$ fraction containing 2 mg. of protein/ml.], 1 μmole of sodium glyoxylate and water to 1 ml. The reaction was started by addition of enzyme. CoA was omitted from the blank cell.

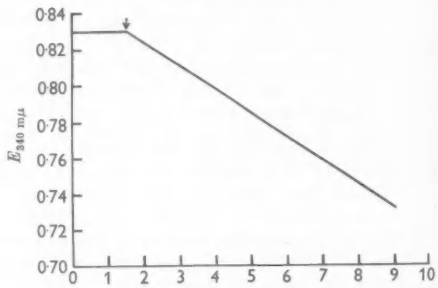


Fig. 6. Reduction of oxalyl-CoA by TPNH. The reaction was followed by change of extinction at 340 mμ in a Cary recording spectrophotometer. The complete reaction system, contained in 3 ml. quartz cells, consisted of: 75 μmoles of sodium pyrophosphate buffer (pH 7·5), 2 μmoles of cysteine, 10 μmoles of ATP, 0·5 μmole of CoA, 1·0 μmole of succinate, 10 μmoles of potassium oxalate, 0·5 μmole of TPNH (omitted from blank cell), 0·1 ml. of enzyme extract [0–80% $(NH_4)_2SO_4$ fraction] and water to 3·0 ml. Time of addition of the oxalate is indicated by the arrow.

of two molecules of glyoxylate (Quayle & Keech, 1959*a*, 1960):

$$2\,CHO{\cdot}CO_2H \rightarrow \begin{bmatrix} CO_2H \\ | \\ CH{\cdot}OH \\ | \\ CHO \end{bmatrix} + CO_2 \qquad (10)$$

Formation of C_4 acids

The activities of malate synthetase, *iso*citratase and condensing enzyme in extracts of *P. oxalaticus* were measured by the procedures of Dixon & Kornberg (1959). The activities of the first two enzymes were very low, i.e. 0·48 μmole of acetyl-CoA utilized/hr./mg. of protein for malate synthetase and 0·10 μmole of glyoxylate formed/hr./mg. of protein for *iso*citratase. These values compare with 12·4 μmoles of acetyl-CoA utilized/hr./mg. of protein for condensing enzyme.

The low activity of malate synthetase suggests that C_4 acids in oxalate-grown *P. oxalaticus* are made primarily by some route not involving this enzyme. One probable mechanism is carbon dioxide fixation by pyruvate or phosphopyruvate, and enzymes catalysing such reactions were found in the formate-grown organism (Quayle & Keech, 1959*c*). When extracts of oxalate-grown *P. oxalaticus* were incubated with [^{14}C]bicarbonate, rapid incorporation of isotope into malate, aspartate and tricarboxylic acid cycle intermediates was

Table 8. *Fixation of* [^{14}C]*bicarbonate in the presence of pyruvate and phosphopyruvate*

The reaction mixture contained 20 μmoles of tris buffer (pH 7·3), 60 μmoles of $NaH^{14}CO_3$ (20 μc), additions as indicated, 0·05 ml. of bacterial extract (prepared in a Hughes press) and water to a final vol. of 0·60 ml. The reaction was started by addition of extract and the mixture incubated at 30° for 15 min. The reaction was stopped by adding 2 ml. of hot ethanol; the resulting precipitates were centrifuged down and discarded. The supernatants were concentrated, the contents were chromatographed two-dimensionally, and the radioactive compounds, located by radioautography, were assayed for radioactivity *in situ*.

Additions	Radioactivity (counts/min.)					
	Malate	Glutamate	Serine	Citrate	Aspartate	Fumarate
None	30	0	0	0	33	0
Pyruvate (5 μmoles)	79	20	0	20	0	0
Pyruvate (5 μmoles) + DPNH (10 μmoles)	33	0	0	20	0	0
Pyruvate (5 μmoles) + TPNH (10 μmoles)	394	0	0	25	0	0
PEP (3 μmoles)	3420	460	470	255	1790	970
PEP (3 μmoles) + IDP (0·28 μmole)	3140	0	0	183	6400	725

found when the incubation was carried out in the presence of phosphoenolpyruvate (PEP) (Table 8). This indicated either the presence of a PEP carboxylase (Bandurski & Greiner, 1953) catalysing the irreversible carboxylation of PEP:

$$PEP + CO_2 \rightarrow \text{oxaloacetate} + HPO_4^{2-} \quad (11)$$

or a nucleoside diphosphate-dependent oxaloacetate carboxylase (Utter & Kurahashi, 1954):

$$PEP + CO_2 + IDP \rightleftharpoons \text{oxaloacetate} + ITP \quad (12)$$

(IDP, inosine diphosphate; ITP, inosine triphosphate) on both enzymes. The stimulation of total fixation on addition of inosine diphosphate is evidence for the existence of the last-named enzyme. The presence of a 'malic enzyme' (Ochoa, Mehler & Kornberg, 1948) catalysing the reductive carboxylation of pyruvate:

$$\text{Pyruvate} + CO_2 + TPNH + H^+ \rightleftharpoons \text{malate} + TPN^+ \quad (13)$$

was suggested by the fixation of [^{14}C]bicarbonate into malic acid in the presence of pyruvate and TPNH. DPNH was not active in this system.

DISCUSSION

The work of Jakoby *et al.* (1956) on the decarboxylation of oxalate by a bacterial enzyme system provided the first evidence for the existence of oxalyl-CoA. The work reported in this paper shows that extracts of *P. oxalaticus* also catalyse the formation of this compound. In addition, oxalate is decarboxylated by a similar series of reactions. It is possible that a re-examination of the system of Jakoby *et al.* (1956) would show the two respective reaction sequences to be identical. The overall decarboxylation rates recorded by Jakoby and co-workers are very low compared with those obtained from *P. oxalaticus*. Since the former authors have not recorded figures for the actual amounts of enzyme extract used to catalyse the decarboxylation, it is not possible to see if the relatively low decarboxylation rates are merely due to the use of protein concentrations lower than have been used in the present study. Comparison of the stoicheiometric requirements of the two reaction systems suggests, however, that the cofactors used by Jakoby *et al.* may have effected only a partial reconstitution of full enzymic activity. These workers showed a requirement for substrate quantities of ATP and catalytic quantities of acetate, CoA, TPP and magnesium ions; the ATP, acetate and CoA requirements could be met with substrate quantities of acetyl-CoA or acetyl phosphate. This compares for *P. oxalaticus* with the requirements for catalytic amounts of ATP, succinate, CoA, TPP and magnesium ions; catalytic quantities of succinyl-CoA replace the succinate, ATP and CoA requirement. This suggests the operation of a cyclic mechanism for the decarboxylation of oxalate to formate, in which the CoA grouping from formyl-CoA is transferred back to oxalate by transferase enzyme(s) either directly, or indirectly via succinate (see Fig. 7). A similar type of cycle has been discovered in *Pseudomonas* species for the decarboxylation of malonate to acetate by Hayaishi (1954) and Wolfe & Rittenberg (1954). As will be discussed further, the oxidation of formate probably represents the main energy-yielding reaction of the organism while growing on oxalate, and thus the expenditure of substrate quantities of ATP in converting oxalate into formate could lead to the loss of 30 % of the possible energy yield in the complete oxidation of oxalate to carbon dioxide. It seems more reasonable to expect the alternative cyclic mechanism to operate, and hence, substitution of succinate for acetate in the system of Jakoby *et al.* (1956) might well show the decarboxylation to proceed in an identical fashion with that catalysed by extracts of *P. oxalaticus*.

Oxalylhydroxamate could only be isolated in trace amounts during the decarboxylation of oxalate by extracts of *P. oxalaticus*, indicating a very rapid turnover of small quantities of oxalyl-CoA or instability of the compound or a combination of both factors. Evidence from the reversal of the oxalyl-CoA reductase indicates the instability of the compound at pH 8·5, although it is not yet known if its decomposition is spontaneous or enzymic.

The detection of formylhydroxamate, also in small amounts, is evidence for the existence of formyl-CoA, the formation of which has previously been suspected in extracts of *C. kluyveri* (Stadtman, 1953; Lieberman, 1954) and an oxalate-grown organism (Jakoby *et al.* 1956).

The reduction of oxalyl-CoA by TPNH to glyoxylate in *P. oxalaticus* is taken to be the first reductive step in the incorporation of oxalate into cell constituents. The fact that no such enzyme activity could be found in formate-grown *P. oxalaticus* indicates that this enzyme is under adaptive control and is necessary for the metabolism of oxalate in this organism. The reaction is another example of the reduction of a carboxylic acid group to an aldehyde group via, presumably, an intermediate CoA thioacetal. This has been shown to occur in *C. kluyveri* (Burton & Stadtman, 1953) and in *E. coli* (Dawes & Foster, 1956) where acetyl-CoA is reduced to acetaldehyde with DPNH. The reduction of palmityl-CoA with TPNH in the synthesis of sphingosine by rat-brain tissue (Brady & Koval, 1958), and β-hydroxy-β-methylglutaryl-CoA to mevalonic acid with TPNH by yeast (Ferguson, Dunn & Rudney, 1959), are further examples of this type of reaction.

No evidence has been found for the presence of a direct pyridine nucleotide-linked oxalic dehydrogenase, although a DPN-linked formic dehydrogenase is present. The burst of carbon dioxide before oxygen uptake observed during oxidation of oxalate by washed suspensions of *P. oxalaticus* suggests that in fact oxidation of oxalate proceeds via initial decarboxylation to formate. The formate, on being oxidized by the formic dehydrogenase, could then serve as primary source of reducing power necessary for synthesis of ATP and reduction of oxalate. In order to transfer the reducing power from DPNH to TPN, a transhydrogenase must be present. The fact that [^{14}C]oxalate is reduced to [^{14}C]glycerate by crude cell-free extracts in the presence of acetyl-CoA and DPNH (Quayle & Keech, 1959*a*, 1960), despite the fact that the oxalyl-CoA reductase is now shown to be strictly TPN-specific, is evidence for the presence of such a transhydrogenase. Evidence for the reverse reaction, reduction of DPN by TPNH, was found during assay of the extract for reduced pyridine nucleotide–cytochrome *c* reductase activity. With the assay system described by Brodie (1955), it was found that DPNH reduced cytochrome *c* four times as fast as TPNH did in crude sonic extracts of *P. oxalaticus*. The reduction by TPNH was doubled by the addition of equimolar quantities of DPN, suggesting a reduction of the DPN by the TPNH.

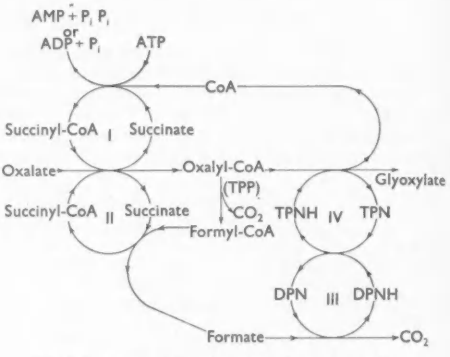


Fig. 7. Conversion of oxalate into glyoxylate.

The assignment of formate as the main source of reducing power for the organism allots a role for the oxalate-decarboxylation cycle (see Fig. 7) in terms of generation of formate. Oxalyl-CoA is then situated at the branching point of two reaction sequences: (*a*) decarboxylation to formate, which is the source of DPNH; (*b*) reduction by TPNH to glyoxylate, which initiates the synthesis of cell constituents.

A major factor controlling these two alternative pathways is probably the relative levels of the reduced and oxidized pyridine nucleotides. This would enable a sensitive balance between synthesis and breakdown to be maintained.

The oxidation of oxalate to carbon dioxide would involve cycles II and III, the sum of which is

$$\text{Oxalate} + \text{DPN}^+ \rightarrow 2\text{CO}_2 + \text{DPNH} + \text{H}^+ \quad (14)$$

The coupling of the oxidation of oxalate to the synthesis of glyoxylate would involve cycles I–IV, the sum of which is

$$2\ \text{Oxalate} + \text{ATP} \rightarrow \text{glyoxylate} + 2\text{CO}_2 + \text{AMP} + \text{P}_i\text{P}_i \text{ or } (\text{ADP} + \text{P}_i) \quad (15)$$

(P_iP_i, inorganic pyrophosphate; P_i, inorganic orthophosphate)

The need for ATP is presumably met by phosphorylation of ADP coupled to the oxidation of more oxalate.

Further stages in the conversion of glyoxylate into cell constituents may take place by a scheme

similar to that proposed by Kornberg & Gotto (1959) for the metabolism of glycollate by a glycollate-grown organism in which two molecules of glyoxylate are condensed to one molecule of a C_3 compound and one molecule of carbon dioxide; the C_3 compound (probably tartronic acid semialdehyde) is then reduced and phosphorylated to 3-phosphoglyceric acid. Evidence for the occurrence of these reactions in *P. oxalaticus* has previously been presented (Quayle & Keech, 1960). Since only very low activities of malate synthetase have been detected in oxalate-grown *P. oxalaticus*, whereas both a TPN-linked 'malic enzyme' and an inosine diphosphate-stimulated PEP carboxylase are present, it is likely that C_4 acids are made by carbon dioxide fixation with pyruvate or PEP rather than through condensation of acetyl-CoA and glyoxylate by malate synthetase.

Work is currently in progress on the further purification of oxalyl-CoA reductase.

SUMMARY

1. Reactions of oxalate, glyoxylate, formate and bicarbonate in cell-free extracts of *Pseudomonas oxalaticus* (OXI) grown on oxalate have been studied.

2. The extracts catalyse a decarboxylation of oxalate to formate which is dependent on catalytic quantities of succinate, adenosine triphosphate, coenzyme A and thiamine pyrophosphate. Magnesium ions and reduced glutathione accelerate the reaction. Succinate, adenosine triphosphate and coenzyme A can be replaced by catalytic quantities of succinyl-coenzyme A. The extracts catalyse the formation of succinyl-coenzyme A from succinate but no similar direct activation of oxalate, formate or acetate has been detected.

3. During the decarboxylation of oxalate under the above experimental conditions both oxalylhydroxamic acid and formylhydroxamic acid have been isolated on addition of hydroxylamine, indicating the formation of coenzyme A esters of oxalic acid and formic acid.

4. It is suggested that oxalyl-coenzyme A is synthesized by transferase action between succinyl-coenzyme A and oxalate. Oxalyl-coenzyme A is decarboxylated to formyl-coenzyme A, the coenzyme A grouping of which can be transferred back to oxalate either directly, or indirectly via succinate.

5. Whole cells of *P. oxalaticus* oxidize both formate and oxalate. Oxidation of oxalate is preceded by a rapid evolution of carbon dioxide suggesting that the oxidation proceeds via formate. Cell-free extracts possess a diphosphopyridine nucleotide-linked formic dehydrogenase but no pyridine nucleotide-linked oxalic dehydrogenase was found.

6. Extracts catalyse a triphosphopyridine nucleotide- and coenzyme A-dependent oxidation of glyoxylate to oxalate. The initial reaction product is considered to be oxalyl-coenzyme A which is hydrolysed under the experimental conditions to oxalate. Indirect evidence indicates the reaction to be reversible, and it is suggested that this is the primary reduction reaction in the conversion of oxalate into cell constituents.

7. The extracts incorporate [^{14}C]bicarbonate into malate and citrate in the presence of pyruvate and reduced triphosphopyridine nucleotide. [^{14}C]-Bicarbonate is incorporated mainly into malate and aspartate in the presence of phosphopyruvate; this fixation is stimulated by inosine diphosphate.

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The Kinetics of Deoxygenation of Human Haemoglobin

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Until 1949, kinetic data for the reactions of haemoglobin with oxygen and carbon monoxide, obtained almost entirely by Hartridge & Roughton (1923, 1925), Roughton (1934) and Millikan (1933) in experiments with sheep haemoglobin, were considered to conform to simple first-order and second-order rate equations, and to be consistent, therefore, with the assumption that the four haems in the molecule behave independently. The best equilibrium data, on the other hand, could only be interpreted by the intermediate-compound theory of Adair (1925) with the assumption of haem interaction (Forbes & Roughton, 1931; Courtice & Douglas, 1947). This situation was reviewed by Roughton (1949), who reported that re-inspection of earlier kinetic data, and new experiments on the deoxygenation of sheep haemoglobin in the presence of sodium dithionite (Legge & Roughton, 1950), gave evidence of haem interaction, notably a significant upward trend in the value of the overall first-order velocity coefficient in the early stages of the deoxygenation reaction. This was confirmed with human haemoglobin under various conditions (cf. Dalziel, 1953, 1954), as will be described here. The further evaluation of the intermediate-compound hypothesis has since been pursued with remarkable success by Roughton, Gibson and their collaborators. For sheep haemoglobin the four equilibrium constants for the oxygen reaction (Roughton, Otis & Lyster, 1955) and the four combination velocity constants for the carbon monoxide reaction (Gibson & Roughton, 1957*a*) have been determined to within narrow limits. Little further work has been reported for the deoxygenation reaction, however.

For the investigation of the kinetic effects of haem interaction, the oxygen dissociation appeared to be the reaction of choice, since previous work had indicated that the reverse reaction could be eliminated by an excess of sodium dithionite, and that the analysis of consecutive reactions would be simpler than for the reverse, combination reaction. This was the main purpose of the work reported here; a secondary objective was to compare the kinetic properties of haemoglobin from several normal individuals and from persons suffering from various types of anaemia. The work, which was carried out during 1948–53, forms part of a Ph.D. thesis approved by the University of London, and brief accounts have also been published in the literature (Dalziel, 1954, 1955, 1958). It was found that secondary reactions between dithionite-

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oxidation products and the pigment interfered with the spectrophotometric analysis (Dalziel & O'Brien, 1951, 1952, 1957; cf. Legge & Roughton, 1950), and so far as haem interaction effects are concerned the full benefits of the precision of the experimental technique devised for the work (Dalziel, 1953, 1954) could not be realized. Nevertheless, reasonably accurate kinetic data were obtained for the deoxygenation of human haemoglobin under a wide range of conditions of concentration, acidity and temperature.

EXPERIMENTAL

Kinetic measurements

The rapid-reaction apparatus consists essentially of a constant-flow apparatus of the gas-pressure type in conjunction with a Beckman DU photoelectric spectrophotometer, and has been described in detail elsewhere (Dalziel, 1953, 1954). The mixing chamber and observation tube, of 2 mm. bore, was fed by two jets of 1 mm. bore. Total volume flow rates of 5–10 ml./sec., or linear flow rates of 150–300 cm./sec., were used, and mixing of the two reactant solutions was 99% complete in 5–8 msec. Temperature was controlled to within 0·1°.

Either oxyhaemoglobin (HbO_2) reactant or deoxygenated haemoglobin (Hb) calibration fluid could be run from one jet, and either dithionite reactant or HbO_2 calibration fluid from the other. The reactants and calibration fluids were kept in the storage vessels for 20 min. to allow temperature and pressure equilibration and destruction of peroxide formed by exposure of the dithionite solution to air (Dalziel & O'Brien, 1957). For measurements at the usual wavelength of 430 mμ ($\lambda_{max.}$ of Hb) the procedure at each point of observation was: (1) a little HbO_2 reactant was run through jet 1 to displace Hb calibration fluid containing dithionite; (2) with jet 1 closed, HbO_2 calibration fluid was run from jet 2, whilst the spectrophotometer was adjusted to read $E = 0$; (3) with jet 2 closed, Hb calibration fluid was run from jet 1, and $\Delta E_{100\%}$, the extinction change corresponding to complete deoxygenation, was recorded; (4) the two reactants were run together, and the extinction, ΔE_t, was recorded; (5) operations (1) and (2) were repeated to check the stability of the spectrophotometer.

The percentage saturation of the pigment with oxygen at time t after mixing, calculated from the distance of the point of observation from the mixing chamber and the flow rate, was calculated as $Y = 100\,(1 - \Delta E_t/\Delta E_{100\%})$.

Precision of the measurements. The precision of the spectrophotometric analysis of the reaction mixture (Dalziel, 1953, 1958) depends upon the constancy of the relative delivery rates, and the molar extinction coefficients of HbO_2 and Hb at the wavelength chosen for measurements. The most suitable wavelength is 430 mμ ($\lambda_{max.}$ of Hb), with 415 mμ ($\lambda_{max.}$ of HbO_2) as second choice. The calculated errors of measurements of the percentage saturation of the pigment are, at 430 mμ, $\pm$0·5% at 95% saturation and $\pm$1·1% at 5% saturation, and, at 415 mμ, $\pm$1·9% at 95% saturation and $\pm$0·9% at 5% saturation. The precision is little affected by variation of $\Delta E_{100\%}$ from 0·2 to 1·2. Nominal band widths of 4–6 mμ were used.

Materials

Oxyhaemoglobin. Haemolysates of washed red cells, diluted with buffer solution, were used without further purification. The HbO_2 solutions contained less than 2% of methaemoglobin, as shown by spectrophotometric analysis for cyanmethaemoglobin. The addition of 3% of methaemoglobin did not affect kinetic data in control experiments.

Clear solutions were obtained by haemolysis with saponin, which had no effect on the kinetics in control experiments. Three experiments with the same sample of washed red cells on three successive days gave identical results, but experiments were usually made not later than the day after the blood was withdrawn. With measurements at 430 mμ, 15 ml. of normal blood was sufficient for a kinetic curve of eighteen points.

Sodium dithionite. Commercial samples (British Drug Houses Ltd.) were used. The solid was stored in a sealed bottle. Dry, freely running samples assayed as 95–98% of $Na_2S_2O_4,H_2O$ (Vogel, 1939).

Buffers. Phosphate and borate buffers were prepared according to Clarke and Lubs (Vogel, 1939).

Preparation of reagents

Oxyhaemoglobin reactant. Red cells were separated by centrifuging from whole blood, to which heparin (0·01 g./100 ml.) had been added, and were washed three times with sodium chloride solution (0·9 g./100 ml.). Saponin solution (3 vol., 0·1 g./100 ml.) was added to 1 vol. of packed red cells, and after 10 min. the haemolysate was centrifuged. The clear supernatant solution was diluted with 1500 ml. of buffer solution. The HbO_2 concentration, expressed as m-equiv./l. (mEq), that is m-moles of haem or combined oxygen/l., was calculated from measurements of $E_{576\,m\mu}$ and a value for ϵ_{mEq} of 15·4 (Dalziel & O'Brien, 1954; Lemberg & Legge, 1949).

Dithionite reactant. This solution was prepared with minimal contact with air, and alkali was added to neutralize the acid formed. For the usual concentration of 0·4 g./100 ml., 5 g. was dissolved in 1225 ml. of buffer plus 25 ml. of 0·2M-NaOH solution in a separating funnel, and the solution was immediately covered with a layer of liquid paraffin. After the Hb calibration fluid had been prepared, the remaining dithionite reactant was run into a storage vessel in the kinetic apparatus and covered with 0·5 cm. of liquid paraffin.

Haemoglobin calibration fluid. To 250 ml. of HbO_2 reactant in a 500 ml. volumetric flask, 250 ml. of dithionite reactant was added from the separating funnel, the mixture was quickly poured into the storage vessel with minimal agitation and at once covered with liquid paraffin.

RESULTS

Variation of the initial oxyhaemoglobin concentration

The time required for the deoxygenation of a given fraction of the oxyhaemoglobin, calculated from spectrophotometric analysis at 430 mμ, is independent of the initial oxyhaemoglobin concentration, and the reaction follows an approximately first-order course (Fig. 1). The first-order plot is strictly linear only beyond 60% saturation, how-

ever; the velocity coefficient, defined by $kY = -dY/dt$, increases significantly during the earlier stages of the reaction (see also Table 2). Values for the first-order velocity coefficient calculated over the last 60 % of the reaction, denoted by k_a, are given in Table 1 for a series of experiments at pH 8·4 in which the initial oxyhaemoglobin concentration varies fivefold. Haemoglobin from a single individual was used. When corrected to 18·0° by means of the temperature coefficient given later, the results of these nine experiments show a variation of ± 3·5 %, which confirms the precision of the technique.

Comparison of spectrokinetic measurements at several wavelengths

As a test of the validity of the spectrophotometric analysis, kinetic curves were obtained by measurements at two or more wavelengths with the same blood sample. In the most detailed experiments, with two samples of blood drawn for transfusion and containing citrate and glucose, the five wavelengths of the absorption maxima of oxyhaemoglobin and haemoglobin were used (Fig. 2). Over the whole course of the reaction at pH 8·4 the values of the percentage saturation calculated from measurements at 415 mμ were significantly

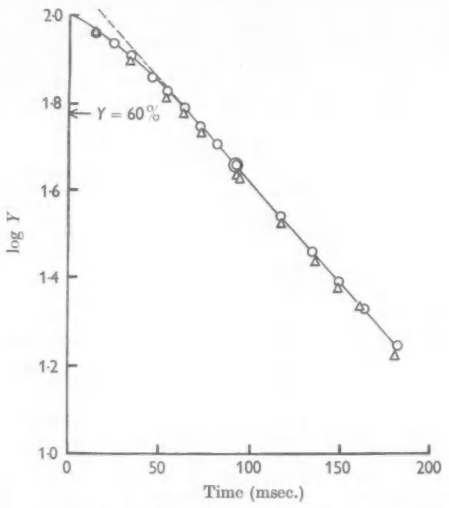

Fig. 1. First-order plots for the rate of deoxygenation of oxyhaemoglobin at two concentrations. Concn. of $Na_2S_2O_4$, 0·2 g./100 ml.; pH 8·4. *Y*, Percentage saturation of the pigment with oxygen. ○, Expt. no. 11, 17·7°, 0·017 m-equiv. of HbO_2/l.; △, Expt. no. 14, 17·8°, 0·067 m-equiv. of HbO_2/l.

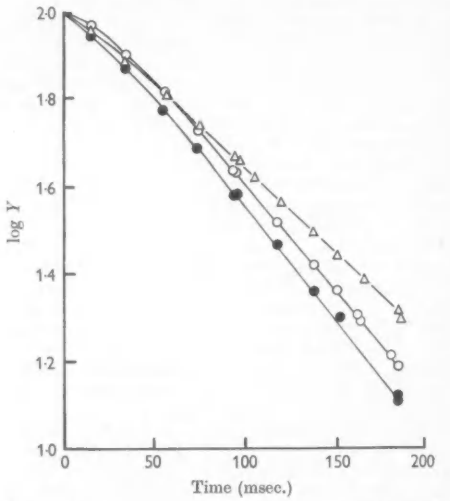

Fig. 2. Kinetic curves recorded at three wavelengths in experiments with the same sample of oxyhaemoglobin: pH 8·4; concn. of $Na_2S_2O_4$, 0·2 g./100 ml.; temp. 18·2°. ○, Expt. 44, 430 mμ, 0·037 m-equiv. of HbO_2/l.; ●, Expt. 45, 415 mμ, 0·068 m-equiv. of HbO_2/l.; △, Expt. 46, 578 mμ, 0·33 m-equiv. of HbO_2/l.

Table 1. *Data of nine experiments with oxyhaemoglobin of a normal individual*

Conditions were: pH 8·4; concn. of $Na_2S_2O_4$, 0·2 g./100 ml.; λ 430 mμ. [HbO_2], concn. of oxyhaemoglobin.

Expt. no.	Flow rate (ml./sec.)	[HbO_2] (m-equiv./l.)	Temp.	k_a (sec.$^{-1}$)	k_a at 18° (sec.$^{-1}$)
9	5·1	0·0325	17·6°	10·2	10·7
10	5·1	0·0380	17·6	10·2	10·7
11	5·1	0·0170	17·7	10·8	11·2
12	5·1	0·079	17·9	11·3	11·4
13	5·1	0·0284	17·7	10·7	11·1
14	5·1	0·067	17·8	10·9	11·2
16	5·1	0·0268	18·4	11·6	11·0
17	9·8	0·0327	18·5	11·8	11·0
19	8·1	0·028	18·5	11·7	10·9
				Mean	11·1 ± 0·4

smaller than those obtained at 430 mμ. The values obtained at 578, 560 and 540 mμ agreed with one another, but were greater over the last half of the reaction than those obtained at 430 mμ. In each case the first-order plot shows a significant increase of slope over the early part of the deoxygenation, but is linear below 60% saturation. Values for k_a calculated from the slopes are 11·8 sec.$^{-1}$ at 415 mμ, 10·8 sec.$^{-1}$ at 430 mμ and 8·9 sec.$^{-1}$ at 578 mμ.

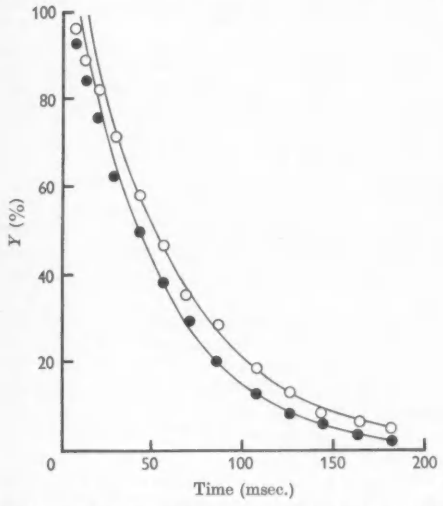

Fig. 3. Time course of deoxygenation at pH 8·4 recorded at 430 mμ (○) and 415 mμ (●). Expt. no. 69, 21·1°, 0·039 m-equiv. of HbO_2/l., concn. of $Na_2S_2O_4$ 0·2 g./100 ml. The lines are calculated first-order curves fitted to the data in the range Y 60–20%.

In all subsequent experiments with fresh blood, under various conditions of concentration, temperature and pH, measurements were made at both 415 and 430 mμ. The results obtained at pH 8·4 (Fig. 3), and at pH 9·5, confirmed those just described. Under all conditions the apparent time course of the reaction was similar at the two wavelengths. The differences between the calculated percentage saturation decreased with decrease of pH and at pH 6·3 identical values were obtained at the two wavelengths (Fig. 4).

These experiments show that the assumption that the reaction mixture contains only two absorbing species, characterized by the absorption spectra of the initial reactant and final product, is not strictly valid at neutral and alkaline pH.

Variation of dithionite concentration. The results in Fig. 5 show that deoxygenation is preceded by an induction period which decreases from about 30 msec. with a dithionite concentration of 0·05 g./100 ml. to less than 5 msec. with dithionite concentrations greater than 0·2 g./100 ml. These experiments were made at pH 8·4 and 18°, with measurements at 430 mμ. Similar results were

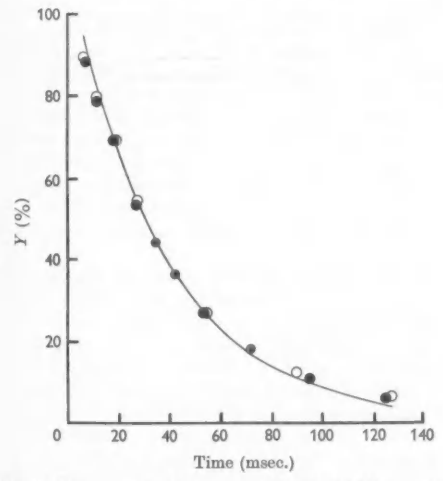

Fig. 4. Time course of deoxygenation at pH 6·3 recorded at 430 mμ (○) and 415 mμ (●). Expt. no. 65, 13·8°, 0·038 m-equiv. of HbO_2, concn. of $Na_2S_2O_4$, 0·2 g./100 ml. The lines are calculated first-order curves fitted to the data in the range Y 60–20%.

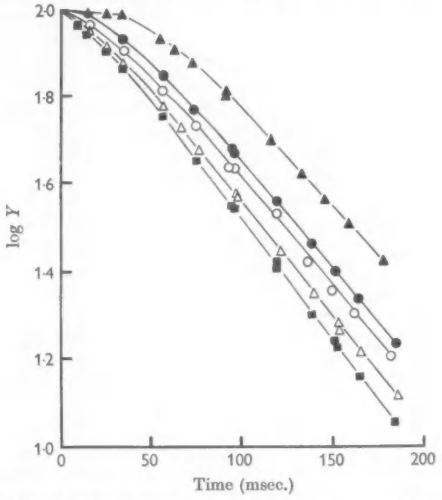

Fig. 5. Kinetic curves at 430 mμ with various dithionite concentrations: ▲, 0·05; ●, 0·1; ○, 0·2; △, 0·4; ■, 0·8 g./100 ml. Expt. nos. 20–24, pH 8·4, temp. 18°, 0·031–0·036 m-equiv. of HbO_2/l.

obtained with measurements at 415 mμ, and also at pH 7·0, where the induction period was shorter than at pH 8·4. The value of k_a increases slightly with dithionite concentration over the range tried, but the upward trend of k during the first 40 % of the reaction persists. This is shown in Table 2 by values of k calculated from pairs of successive measurements in the experiments with dithionite concentrations of 0·05 and 0·8 g./100 ml. A 16-fold increase of dithionite concentration, which practically eliminates the induction period, causes only a small increase in the rate of haemoglobin formation, and does not accelerate the early stages of the reaction, from Y 90 to 60 %, to a greater extent than the later stages, and the initial upward trend in k remains. Similar results were obtained with haemoglobin from another individual, and also with a second sample of sodium dithionite.

In all the following experiments, the dithionite concentration was 0·2 g./100 ml.

Variation of pH. The reaction was studied at several pH values from 5·9 to 9·4, at temperatures from 18 to 21°, and with spectrophotometric measurements at both 415 and 430 mμ. The buffer solutions were Clarke and Lubs borate (pH 8·0–9·4) and phosphate (pH 5·9–7·65), and the ionic strengths of the reaction mixtures varied from 0·1 to 0·17. Experiments could not be made in more acid solutions because of turbidity; even at pH 5·9, the light absorption of the product changed slowly on standing, but at pH 6·3 the product was stable.

Plots of log Y against time were linear from 60 % down to 20 % saturation at every pH value. At pH > 7·0, linearity extended to less than 5 % saturation, but in slightly acid solutions there was evidence of slight deviations towards slower rates below 20 % saturation (cf. Fig. 4). The gradual increase in the first-order velocity coefficient in the early stages of the reaction was evident over the whole pH range, but became progressively less marked with decrease of pH: at pH 6·3, the maximum value k_a was reached at 70 % saturation.

From studies of the effect of temperature on the reaction rate, described below, values for k_a at 18° were calculated, and are plotted against pH in Fig. 6.

Variation of temperature. Values of log k_a obtained from measurements at 430 mμ in experiments with oxyhaemoglobin of one individual, at various pH values and temperatures from 15 to 30°, are plotted against the reciprocal of the absolute temperature in Fig. 7. The activation energy calculated from the slopes increases slightly with increasing acidity, and the difference between the values of 22 000 cal./mole at pH 6·3 and 26 000 cal./mole at pH 8·4 is outside the random error of the measurements.

To test whether the rate during the first 40 % of deoxygenation, during which k increases, varies with temperature to the same extent as the later stages, log Y was plotted against $t.k_a/2{\cdot}3$ for experiments at pH 8·4 in the temperature range 15–30°, over which k_a increases ninefold. The slopes of these plots (Fig. 8) are equal to k/k_a, and their constancy shows that this ratio is independent of temperature. The average value of k/k_a is about 0·7 in the range Y 80–95 %. The curves for the several experiments are not exactly superimposed because differences of flow rate affect the rate of mixing, and temperature differences alter the duration of the induction period.

Table 2. *Variation of velocity coefficient during the deoxygenation of oxyhaemoglobin in the presence of dithionite at* pH 8·4

Velocity coefficient, $k = 2{\cdot}3\,\Delta \log Y/\Delta t$.

Expt. no. 20 0·05 g. of $Na_2S_2O_4$/100 ml.		Expt. no. 23 0·8 g. of $Na_2S_2O_4$/100 ml.	
Mean value of Y (%)	k (sec.$^{-1}$)	Mean value of Y (%)	k (sec.$^{-1}$)
92	6·2	90	7·4
83	7·9	83	10·0
78	6·9	76	9·8
70	8·7	68	11·6
57	9·9	60	12·2
45	10·5	54	12·6
39	9·9	48	12·1
34	10·6	40	12·0
30	10·1	31	12·8
		23	13·2
		18·5	12·8
		16	12·0
		13	12·2

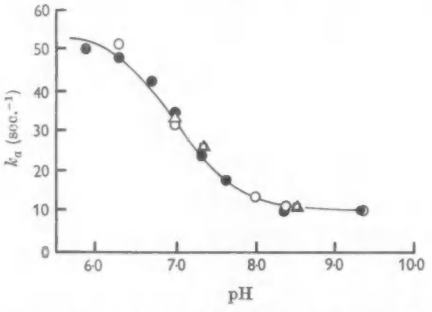

Fig. 6. Variation of the first-order velocity coefficient with pH at 18° for the deoxygenation of oxyhaemoglobin samples from K.D. (●), and three other individuals (○, □, △). The curve was calculated from pK = 7·0, k_a for acidic form = 55 sec.$^{-1}$ and k_a for basic form = 10 sec.$^{-1}$. 0·03–0·04 m-equiv. of HbO_2/l.; concn. of $Na_2S_2O_4$, 0·2 g./100 ml.

Variation of ionic strength. The effect of ionic strength on k_a was studied over a range of pH by varying the buffer concentration and by the addition of sodium chloride. At pH 8·5 and 9·5, the buffer was borate–potassium chloride–sodium hydroxide and, at pH 7·0 and 6·3, sodium dihydrogen phosphate–sodium hydroxide. At pH 8·0, both borate and phosphate buffers were used in two concentrations, 0·05 and 0·2 M. There was no evidence of specific ion effects. With increase of ionic strength, k_a increased at pH 8·0–9·5, decreased at pH 6·3, and varied only slightly at pH 7·0. The results shown in Fig. 9 were obtained from measurements at 430 mμ. The salt effects at pH 6·3 and 9·5 were confirmed in measurements at 415 mμ.

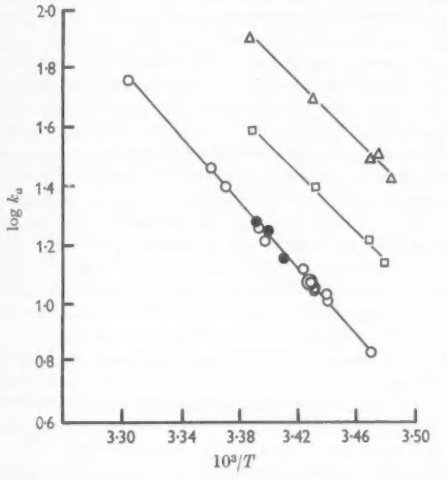

Fig. 7. Arrhenius plots for the deoxygenation of oxyhaemoglobin (K.D.) at several pH values: △, pH 6·3; □, pH 7·35; ○, pH 8·4; ●, pH 9·4. 0·03–0·04 m-equiv. of HbO_2/l.; concn. of $Na_2S_2O_4$, 0·2 g./100 ml.

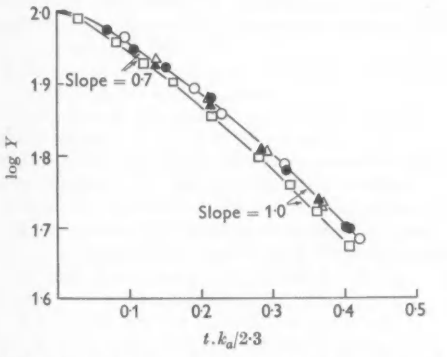

Fig. 8. Plots of log Y against $t.k_a/2·3$ for five experiments at temperatures from 15 to 30°, pH 8·4, 0·03–3·04 m-equiv. of HbO_2/l., concn. of $Na_2S_2O_4$, 0·2 g./100 ml. k_a is the first-order velocity coefficient calculated from measurements in the range Y 60–20 %, and t is time in sec.

Comparative data for oxyhaemoglobin of several individuals. Values for k_a at 18·0° and pH 8·4 for haemoglobin samples from five normal individuals, calculated from measurements at 430 mμ, were 11·1, 11·2, 11·5, 11·8 and 11·8 sec.$^{-1}$. The first of these is the mean of nine experiments (cf. Table 1), and the difference of 6 % between this and the last two values is significant. Some of these samples were also studied at lower pH values (Fig. 6), but for these the differences were within the limits of experimental error. Samples from patients suffering from polycythaemia, untreated pernicious anaemia and acute haemolytic anaemia (acholuric jaundice) gave values of 11·3, 11·4 and 11·3 sec.$^{-1}$ for k_a at pH 8·4 and 18·0°.

Kinetic studies over the pH range 6·5–8·4 were also made with haemoglobin samples from a patient suffering from sickle-cell anaemia and an individual showing sickle-cell trait. These experiments were made at an early stage of the research, and are less reliable than the later work, but the results did not differ significantly from those of control experiments with normal haemoglobin, and showed the same pH effect on the rate and the usual upward trend in k. The values for k_a were smaller, by about 15 %, than those obtained in the later work. This was probably due to the use of a poor sample of dithionite and to neglect of sources of systematic error unsuspected at that time.

Effects of salts related to dithionite. Possible effects of oxidation products and impurities in the dithionite were sought in experiments at pH 8·5. Sodium sulphate, metabisulphite, thiosulphate and

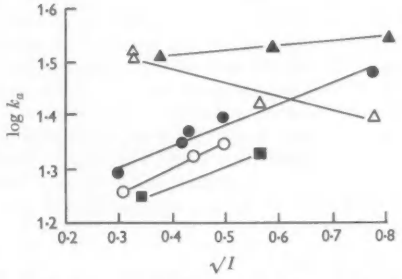

Fig. 9. Variation of the first-order velocity coefficient with ionic strength: ■, pH 9·5; ○, pH 8·5; ●, pH 8·0; ▲, pH 7·0; △, pH 6·3. 0·03–0·04 m-equiv. of HbO_2/l., concn. of $Na_2S_2O_4$, 0·2 g./100 ml.

sulphide added to the dithionite reactant (0·4 g./100 ml.) in concentrations of from 0·08 to 0·2 g./100 ml. had no effect on kinetic measurements at 415 and 430 mμ.

DISCUSSION

The validity of the optical methods which have been used to measure the percentage saturation of haemoglobin with various gases in kinetic studies depends upon the assumption that the reacting mixture contains only two absorbing species, characterized by the absorption curves of the unreacted and fully reacted mixture. This assumption has been tested by the comparison of measurements at several wavelengths, and the results show that it is not strictly true for the deoxygenation reaction in the presence of sodium dithionite. The apparent percentage saturation, after a given time, varied with the wavelength over a range of about 8% in slightly alkaline solutions. Therefore, one or more additional absorbing species must be formed, either as intermediates in the reaction or in secondary reactions. There will in fact be five molecular species in a partially saturated mixture, according to the intermediate-compound theory, but if each of the four haems in the haemoglobin molecule contributes equally and independently to the light absorption, regardless of the presence of a ligand on the iron atoms of the other three, there will be effectively only two absorbing species, oxygenated and unoxygenated haems, and the light absorption should be linearly related to the percentage saturation of the pigment. Hartridge & Roughton (1925) found this to be true within the error of their spectroscopic method of analysis, and it has been stated that tests with more sensitive spectrophotometric methods also support this assumption (Roughton, 1955; Gibson & Roughton, 1957*a*).

The discrepancies between measurements at different wavelengths can be explained by secondary reactions between haemoglobin and hydrogen peroxide formed in the auto-oxidation of dithionite, in which a small proportion of the haemoglobin is irreversibly degraded (Dalziel & O'Brien, 1957). The discrepancies are not large. At every wavelength used, the measurements indicated a similar time course for the reaction, and below about 60% saturation they conformed to the requirements of a simple first-order dissociation. The velocity coefficient, k_a, varied by $\pm 15\%$ about the value obtained at 430 mμ. It is unlikely that the results obtained for the rate of reaction and its variation with temperature, pH and ionic strength are seriously in error.

A reproducibility of $\pm 3{\cdot}5\%$ in k_a was realized consistently in experiments with single blood samples and with samples withdrawn from the same individual at intervals over a period of many months. There were no greater variations between the rates of deoxygenation, at several pH values, of haemoglobin from seven individuals, including three suffering from blood disorders, except at pH 8·4, where the highest and lowest values of k_a, 11·8 and 11·1 sec.$^{-1}$, differed significantly. It is of special interest that no significant differences were detected in the kinetic behaviour, at several pH values, of haemoglobins from a normal individual, one with sickle-cell trait, and a patient suffering from sickle-cell anaemia. Evidently the differences between the globins (Itano & Pauling, 1949) do not affect the haem-linked acidic groups.

In contrast, Gibson, Kreuzer, Meda & Roughton (1955) reported considerable differences between the rates of deoxygenation of three samples of human haemoglobin; for example, at pH 7·1 rate constants of 21, 26 and 36 sec.$^{-1}$ were obtained. Unfortunately, no evidence of the reproducibility of measurements with a single sample of blood was given. Even greater individual variations have, however, been reported for the rates of combination of human and sheep haemoglobin with oxygen and carbon monoxide (Hartridge & Roughton, 1925; Gibson *et al.* 1955; Gibson & Roughton, 1957*a*).

In Table 3, values for k_a at 18° obtained in the present work from measurements at 430 mμ are compared with specific reaction rates for human haemoglobin reported by other workers, corrected where necessary to 18° with the temperature coefficient reported here. The consistency of the data is remarkably good in view of the differences in technique. Millikan (1933) used a constant-flow method with colorimetric analysis, and his results were consistent with a simple first-order course throughout. Dubois (1941) used the deoxygenation of a mixed blood sample as a test of his constant-flow apparatus. Gibson *et al.* (1955) made measurements over the first third of the reaction only, with

Table 3. *Specific rates of deoxygenation of human haemoglobin at* 18°

Source	Specific reaction rate (sec.$^{-1}$)			
	pH 6·0	pH 7·1	pH 8·5	pH 9·1–9·8
Present work, k_a	51	29	11	11
Millikan (1933)	44	24	10	10
Dubois (1941)	—	—	11	—
Gibson, Kreuzer, Meda & Roughton (1955)	—	28	—	11

a stopped-flow method, and the values given in the Table are averages of those reported for three blood samples.

Hartridge & Roughton (1923) found the rate of deoxygenation of sheep haemoglobin to be independent of the dithionite concentration above 0·05 g./100 ml., and concluded that the role of the dithionite is that of a passive oxygen absorbent, which, after an induction period during which the oxygen concentration is reduced sufficiently, permits the dissociation of oxyhaemoglobin and eliminates the reverse reaction. These findings have been generally confirmed here, although the greater precision of the method has revealed an increase of about 10% in the rate of deoxygenation, at pH 8·4, for an increase of dithionite concentration from 0·05 to 0·8 g./100 ml. This small increase can be accounted for as a neutral-salt effect, however. The results establish that deoxygenation by second-order reaction of oxyhaemoglobin with dithionite is very slow, if it occurs at all, but do not of course exclude the possibility that dithionite may have some influence on the rate of dissociation, for example by reaction with the globin.

The variation of the rate of deoxygenation of human haemoglobin with pH is very similar to that of sheep haemoglobin, and can be explained by the assumption (Henderson, 1920) that oxyhaemoglobin contains an oxy-labile acidic group with pK 7·0 and that the deoxygenation of the unionized form is five times as fast as that of the basic form. The corresponding data of Hartridge & Roughton (1923) for sheep haemoglobin were pK 6·7, and a ratio of 7 between the rates of deoxygenation of acidic and basic forms; the differences are within the experimental error. The pH effects on the rates of dissociation of the first carbon monoxide and nitric oxide molecule respectively from the carbon monoxide and nitric oxide compounds of sheep haemoglobin are also accounted for by the assumption of a haem-linked group with pK 6·7 (Gibson & Roughton, 1957*b*, *c*). These kinetic effects may all be attributed to the weaker of the two haem-linked groups revealed by pH effects on the dissociation curve (Ferry & Green, 1929) and differential titration of oxyhaemoglobin and haemoglobin (German & Wyman, 1937), for which pK values of 6·7 and 5·8 have been estimated for horse haemoglobin (Wyman & Ingalls, 1941; Wyman, 1948).

Salt effects on the rate of deoxygenation have not been recorded hitherto. The increase of rate with ionic strength at pH 8·4 and 9·4, and the reverse effect at pH 6·3, cannot be explained by a secondary salt effect on the dissociation of a haem-linked acidic group. A difference of total charge between the oxyhaemoglobin molecule and the activated complex is not consistent with the fact, referred to below, that the entropy of activation appears to be greater in alkaline solution than in acid solution, and the absence of any evidence that the isoelectric points of oxyhaemoglobin and haemoglobin differ significantly. A more likely explanation of the salt effects, perhaps, is that disorganization of the protein structure accompanies the formation of the activated complex (cf. Eley, 1943) and that concomitant redistribution of charge and fission of hydrogen bonds is influenced by the relatively high salt concentrations used here. The situation is complicated by the fact that the processes involved in haem interaction may also be influenced by salts. It would be of interest to investigate salt effects on the dissociation of the first of the four oxygen molecules alone, the rate of which can be determined in the absence of dithionite (Gibson & Roughton, 1955), and also to make similar studies of the deoxygenation of myoglobin, in which haem interaction cannot occur.

The activation energies for the overall deoxygenation, 22 000 cal. at pH 6·3 and 26 000 cal. at pH 8·4, agree very well with the value of 22 000 cal. obtained by Hartridge & Roughton (1923) for sheep haemoglobin at pH 9·0. The latter workers also recorded some measurements at pH 5·6 which indicated a slightly greater activation energy of 25 000 cal., but the difference, which is evidently within the experimental error, was not commented upon. Nevertheless, Eley (1943) regarded the difference as significant, and, on the basis of the transition-state theory, calculated entropies of activation of 36 and 24 cal./degree/mole at pH 5·6 and pH 9·1 respectively. The pH effect on the activation energy indicated by the present experiments, which give entropies of activation of 25 cal./degree/mole at pH 6·3 and 36 cal./degree/mole at pH 8·4, is in the opposite direction, and suggests that the higher rate of deoxygenation in acid solution is due to a lower activation energy and not to a larger positive entropy of activation, as Eley concluded for sheep haemoglobin.

Evidence that the deoxygenation of sheep oxyhaemoglobin does not follow a simple first-order course throughout, as was concluded by Millikan (1933), was first obtained by Legge & Roughton (1950). Their conclusion that an initial upward trend in the velocity coefficient is a genuine feature of the kinetics of the overall reaction is strongly supported by the present experiments, in which the trend is far outside the experimental error and has been demonstrated under various conditions. Experiments in which the dithionite concentration was varied 16-fold, the initial oxyhaemoglobin concentration sixfold, and the flow rate twofold, together with experimental controls of the tech-

nique itself which included kinetic studies of the dehydration of carbonic acid (Dalziel, 1953), established that the effect is not due to insufficiently rapid removal of dissolved oxygen by the dithionite, incomplete mixing, flow anomalies or other technical errors. It is especially important, in view of the occurrence of secondary reactions which interfered with the colorimetric analysis by Legge & Roughton (1950), that the deviations from a simple first-order course have been recorded by measurements with monochromatic light of five wavelengths, comprising the absorption maxima of reactant and product. On the intermediate-compound theory, this feature may be attributed to the fact that k_4, the velocity constant for the dissociation of the first of the four oxygen molecules from oxyhaemoglobin, is smaller, relative to the rate constants for the three succeeding dissociations, than would be expected on a statistical basis. This interpretation is consistent with the equilibrium data (Roughton, 1949), and convincing kinetic evidence in its support was obtained by Gibson & Roughton (1955).

Direct measurements of the individual constant k_4, by a method which does not involve the use of dithionite, have now been reported for both human and sheep haemoglobin, and preliminary comparisons of these data with the overall kinetics in the presence of dithionite have been made (Gibson & Roughton, 1955; Gibson *et al.* 1955). The present findings will be briefly considered in the light of these discussions. First, the overall velocity coefficient k_a is greater at pH 7·1, by a factor of 2·5, than at pH 9·1. Over this pH range, the overall velocity coefficient in the very earliest stages of the reaction is estimated, by extrapolation to zero time, as $< 0{\cdot}6\ k_a$, and should be equal to $0{\cdot}25\ k_4$. On the other hand, direct measurements showed that k_4 is independent of pH over this range. Although this is not inconsistent with the marked pH effect on k_a, which could be put down to an effect on one or more of the other three individual rate constants, the initial upward trend of k should in this case be considerably more marked at pH 7·1 than at pH 9·1. There was no evidence of this in the present experiments; indeed there were some indications of the reverse effect.

Secondly, the finding that the initial trend in the overall velocity coefficient is not significantly altered by temperature change, from 15° to 30°, indicates that the activation energy for k_4 is not greatly different from that calculated for k_a. In this case, the overall data are consistent with direct determinations of k_4: for human haemoglobin at pH 7·1, Gibson *et al.* (1955) obtained an activation energy of 19 600 cal. for k_4, compared with the value of 23 000 cal. for k_a found here. The difference is not great enough to produce a significant temperature effect on the time course of overall deoxygenation.

It is evident that further direct comparisons of the overall deoxygenation kinetics and direct measurements of k_4 on the same blood sample are needed. The value of k_4 appears to vary considerably from sample to sample (Gibson *et al.* 1955), but the reproducibility of the data for a single sample has not been demonstrated. Secondary reactions accompanying the overall deoxygenation in the presence of dithionite have so far discouraged attempts to evaluate all four individual dissociation velocity constants, as has been done for the carbon monoxide reaction (Gibson & Roughton, 1957*a*). These secondary reactions may be minimized by using high concentrations of pigment and dithionite (Dalziel & O'Brien, 1957), and controlled by making measurements at two or more wavelengths corresponding to the absorption maxima of haemoglobin and oxyhaemoglobin. It may also be possible to obtain evidence of the nature and magnitude of the errors from this source by spectrokinetic studies of the reduction of methaemoglobin by dithionite in the presence and absence of dissolved oxygen, since the absorption spectrum of methaemoglobin in the Soret region is similar to that of oxyhaemoglobin.

It is unlikely, however, that the marked differences between the pH effects on k_a and k_4 can be due to secondary reactions of the type demonstrated so far. Dithionite may affect the rate of deoxygenation by reaction with the globin (cf. Gibson & Roughton, 1955). It is known that dithionite can react with amino groups, and Roughton (1944) has suggested that the weaker of the two oxy-labile groups may be an amino group which can bind carbon dioxide. In this connexion studies of the effect of carbon dioxide on the kinetics of deoxygenation would be of interest.

SUMMARY

1. Spectrokinetic studies of the deoxygenation of human haemoglobin in the presence of sodium dithionite have been made under various conditions with a constant-flow rapid-reaction apparatus and a photoelectric spectrophotometer. A reproducibility of $\pm 3{\cdot}5\%$ in estimates of the first-order velocity coefficient has been demonstrated.

2. No significant differences were detected between the kinetic behaviour at several pH values of oxyhaemoglobin samples from normal individuals and from persons suffering from several types of anaemia, including sickle-cell anaemia. The greatest difference observed between two haemoglobin samples was 6% in the specific reaction rate.

3. Significant differences between the results of analyses of the reaction mixture, by measurements

of light absorption at several wavelengths, show that the deoxygenation is accompanied by secondary reactions involving the pigment. The errors thereby introduced are not large, but realization of the full benefits of the precision of the technique in the interpretation of the detailed time course of the reaction is restricted.

4. The time course of the overall reaction cannot be accurately described by the simple first-order relation, $d[HbO_2]/dt = k[HbO_2]$. The calculated value of k increases from an initial value of $< 0{\cdot}6k_a$ to a constant value of k_a when reaction is 40 % complete. This initial trend in k is not affected by wide variations of the initial oxyhaemoglobin and dithionite concentrations, and is evident in measurements at five wavelengths at pH 8·4 and two wavelengths at pH 6·3–9·5, and is therefore considered to be a genuine kinetic feature of the deoxygenation. This confirms the conclusion of Legge & Roughton (1950) for the deoxygenation of sheep haemoglobin, and is in agreement with other recent work on the kinetics and equilibria of sheep haemoglobin and with the intermediate-compound theory.

5. Variations of the rate of deoxygenation with pH are consistent with the presence of an oxylabile group with pK 7·0. The specific reaction rate is five times as great at pH 6·0 as at pH 8·5–9·5. There was no evidence of a pH effect on the initial upward trend in the specific reaction rate.

6. Activation energies of 22 000 cal. at pH 6·3 and 26 000 cal. at pH 8·4 have been calculated from the variation of the rate of deoxygenation with temperature. There was no evidence that the early and late stages of the overall deoxygenation were differently affected by temperature change.

7. With increase of ionic strength, the specific reaction rate decreases at pH values below 7·0 and increases at pH values above 7·0.

8. The results are considered in relation to other recent work, and certain anomalies are discussed.

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The Absorption Spectra, Magnetic Moments and the Binding of Iron in some Haemoproteins

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In the iron porphyrin complexes four co-ordination positions in a plane around the iron atom are occupied by the nitrogen atoms of a porphyrin, and the other two positions are filled by simple monodentate ligands such as water and ammonia. We symbolize the iron porphyrin complexes as FePXY, where P is the porphyrin and X and Y are the co-ordinated groups in the fifth and sixth positions. The relationships between the properties of the groups X and Y and the properties they induce both in porphyrin and simpler iron complexes have been outlined in earlier papers (Williams, 1956, 1959*a*). In this paper we extend and systematize these relationships, and are then able to set up diagnostic rules permitting the recognition of the nature of unknown ligands X and Y from an examination of the properties of their iron porphyrin complex. We use these rules to discuss the co-ordination of the iron atom in a number of haemoproteins. The latter are iron porphyrin complexes in which the fifth co-ordination position at least, and possibly the sixth position also, is occupied by a donor group which is also bound to the protein directly. In the following paper we go on to consider the primary compounds of peroxides with catalase and peroxidase.

Ferric porphyrin complexes

It is generally recognized that there are two main classes of ferric porphyrin complexes (Pauling, 1948). The members of one class have spin moments approximately that of five unpaired electrons, spin 5/2, and magnetic moment 5·92β (Bohr magneton), whereas the members of the other class have magnetic moments approaching that of one unpaired electron, spin 1/2, and magnetic moment 1·73β. Complexes in the first group will be called high-spin complexes, and those in the second group will be called low-spin complexes. In earlier literature the classification 'ionic' and 'covalent' has been used (Pauling, 1931) as well as the classification 'weak field' and 'strong field' (van Vleck, 1935). As both the older classifications imply something about the character of the binding of the iron in the complexes which is inferred without certainty from the magnetic characteristics, it appears preferable to classify the complexes on the basis of the experimental magnetic data. In some $Fe^{III}PXY$ complexes, equilibria exist between the two spin forms (Williams, 1956, 1959*b*; George, Beetlestone & Griffith, 1959). There is a suggestion that a third form of spin 3/2 (three unpaired electrons) is found in a very limited number of cases, e.g. in phthalocyanines (Griffith, 1958). Unfortunately, except for the purely high-spin and purely low-spin $Fe^{III}PXY$ complexes, the magnetic moment arrived at from susceptibility measurements does not uniquely distinguish among the possible configurations. Paramagnetic resonance data, which are generally less ambiguous, are not always obtainable because of experimental difficulties. It would be useful then if the absorption spectra of ferric porphyrin complexes, which are easily measured on small quantities of material, could be analysed to determine the spin state of the Fe^{3+} ion. We examine this possibility below.

Classification of absorption bands of ferric porphyrin complexes. High-spin ferric porphyrin complexes have distinctly different spectra from other metal porphyrin complexes. The particular differentiating characteristics of $Fe^{III}PXY$ spectra are two new absorption bands, one at about 600–650 mμ and a second at 450–500 mμ. Similarly situated bands appear in simpler high-spin ferric complexes with unsaturated anions, e.g. those of 8-hydroxyquinoline and acetylacetone. On this basis we have attributed these bands to properties of the high-spin Fe^{3+} ion in combination with unsaturated ligands (Williams, 1956; Tomkinson & Williams, 1958). We have further suggested that the two bands are charge-transfer bands. The reasons for this diagnosis are as follows.

A charge-transfer band is associated with an electron excitation in which the electron is partially or completely transferred from the vicinity of one atom or group of atoms to another such group. In the excitation there is a considerable change of dipole moment and the transition is therefore allowed and will have a high intensity. For

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example, in ferric thiocyanate the excitation process is thought to be

$$[Fe^{3+}\text{–}S\bar{C}N] \rightarrow [Fe^{2+}\text{–}S\dot{C}N].$$

If this description is extended to ferric complexes with aromatic ligands, then the introduction of substituents in the aromatic rings should have predictable effects upon the absorption spectrum. Electron-donor substituents should facilitate electron transfer and thus cause the band to move to longer wavelengths, whereas electron acceptors should shift the absorption band to shorter wavelengths. This has been observed in the intense bands of ferric 8-hydroxyquinoline complex (Tomkinson & Williams, 1958). On the other hand, the absorption bands in the visible spectrum of ferric complexes with saturated ligands (other than those co-ordinating through sulphur) such as fluoride, acetate or water are very weak. These bands are not charge-transfer bands. Ferric porphyrin complexes $Fe^{III}PXY$ can be prepared with a variety of such simple co-ordinating groups X and Y as fluoride and acetate. The charge-transfer bands which are observed in these ferric porphyrin complexes must be due to electron transfer to the Fe^{3+} ion from the porphyrin dianion and not from the small anions X and Y.

Both high- and low-spin ferric porphyrin complexes also show the group of bands γ (Soret), β and α typical of all metal porphyrins. The bands are due to transition of the π-electrons of the porphyrin (Longuet-Higgins, Rector & Platt, 1950). However, the intensity of the α- and β-bands is low in high-spin ferric porphyrin complexes and the bands are often obscured by the charge-transfer bands. The position of these bands is also affected, although not greatly, by the spin state of the central cation. The low-spin ferric porphyrin complexes do not show charge-transfer bands, and these bands are absent too in low-spin ferric complexes with simpler unsaturated ligands. Equilibrium mixtures of high-spin and low-spin states have spectra which are additive mixtures of two components (Williams, 1956, 1959*b*; George *et al.* 1959).

Positions of the absorption bands of ferric porphyrin complexes. Table 1, which gives the positions and intensities of the bands of a large number of ferric haemoprotein complexes, shows the effect of changes of ligand in the sixth co-ordination position. The fifth position is occupied by a co-ordinating group which is attached to the peptide chain of the protein. In metmyoglobin and methaemoglobin the fifth position is occupied by a nitrogen atom of the imidazole group of a histidine residue. In catalase and peroxidase the groups are not known. The first and fourth bands, the charge-transfer bands, move to shorter wavelength in the order water $\geqslant$ azide $>$ formate $\geqslant$ acetate $>$ fluoride $>$ hydroxide. With the exception of azide this order is closely related to the complex-forming ability of these ligands in simple ferric complexes, shown in Table 2. Charge transfer, in which an electron is transferred from the porphyrin in a complex $Fe^{III}PXY$ to the ferric cation, will be the more difficult energetically the greater the donation to the iron of electrons from X and Y and the greater their negative charge/anion-radius ratio, i.e. the greater the electron density already in the vicinity of the ferric ion. These two factors largely dominate the stability of ferric complexes. Thus the correlation between stability and the position of the charge-transfer bands is hardly surprising. Azide does not conform to the pattern and it is notably different from the other anions in that it is unsaturated. The binding of unsaturated ligands to cations is different from that of saturated ligands. The charge-transfer bands in thiocyanate and cyanate methaemoglobin are also at longer wavelengths than those of the complexes of saturated anions.

The charge-transfer bands of $Fe^{III}PXY$ in which both X and Y are neutral molecules are also at longer wavelengths than when X or Y is a saturated anion. This is shown by simple methaemoglobin and metmyoglobin in which the ligands are water and histidine, and by the hydroxylamine complex of methaemoglobin for which the charge transfer bands are at $\sim 625\,m\mu$ and $490\,m\mu$ (inflexion) (Banaschak & Jung, 1956).

The α-, β- and Soret bands move much less with ligand than do the charge-transfer bands, unless there is a change of spin state. Upon such a change, the Soret band moves to longer wavelengths the lower the spin. In fact, for a series of anions, there is a linear relation between the paramagnetic susceptibility and the wavelength of the peak of the Soret band (Scheler, Schoffa & Jung, 1957). The complexes $Fe^{III}PXY$, in which both X and Y are neutral molecules, do not fit the relationship, the shift being in the same direction but of only half the size. The α- and β-bands move to shorter wavelengths with neutral bases, e.g. imidazole and ammonia, than with anions, e.g. hydroxide and cyanide.

Intensity of the absorption bands of ferric porphyrin complexes. The Soret band does not vary greatly in intensity among the $Fe^{III}PXY$ complexes. This suggests that if 'complexes' are prepared in which a large drop in the intensity of this band is observed, then, in all probability, an attack has taken place on the porphyrin ring rather than a simple change in the co-ordination to the iron (see following paper). The α- and β-bands are more intense in low-spin than in high-spin complexes (see Table 1). The intensity of the α-band in low-spin complexes is very dependent on

Table 1. *Spectroscopic and magnetic characteristics of complexes of some ferric haemoproteins*

The positions of the maxima of the absorption bands ($\lambda_{max.}$) are given in mμ and the millimolecular extinction coefficients (ϵ_{mM}) are per haematin. The columns of charge-transfer bands are headed C.T. The wavelength at which an inflexion in the spectrum occurs is indicated by placing the position in brackets and writing infl. below, or by the latter only if the position is obscure. The magnetic moments are given in Bohr magnetons, β.

Complex		Absorption bands C.T.	α	β	C.T.	γ	Magnetic moment (β)	Reference
Methaemoglobin (horse)								
Fluoride	$\lambda_{max.}$	605	—	(550)	483	403	5·76	1, 2
	ϵ_{mM}	10·9		infl.	10·3	144		
Water pH 6·4		631	(580)	(540)	500	405	5·65	1, 2
		4·4	infl.	infl.	10·0	179		
Acetate		620	575	(540)	497	404	5·44	1
		5·5	5·2	infl.	10·5	178		
Formate		620	570	(540)	496	404	5·44	1
		5·8	5·3	infl.	9·2	178		
Hydroxide		(600)	575	540	(480)	410	4·66	1, 2
		infl.	9·2	11·0	infl.	120		
Ammonia		None	(563)	535	(483)	411	2·93	3, 4
			infl.	10·8	infl.	126		
Imidazole		None	560	534	None	411	2·87	1
			12·5	14·7		105		
Cyanide		None	None	540	None	419	2·50	1, 2
			infl.	12·5		124		
Azide		(630)	575	540	None	417	2·35	1
		infl.	9·9	12·8		134		
Metmyoglobin (horse)								
Fluoride	$\lambda_{max.}$	604	(585)	(550)	487	406	5·77	1, 5
	ϵ_{mM}	9·2	infl.	infl.	9·9	146		
Water pH 6·4		630	(580)	(530)	502	408	5·73	1, 5
		3·9	infl.	infl.	10·2	188		
Hydroxide		(600)	585	539	(490)	411	5·04	1, 5
		infl.	7·8	8·8	infl.	119		
Azide		(635)	570	540	None	420	3·30	1
		infl.	8·7	11·2		123		
Cyanide		None	None	540	None	422	1·96	1
			infl.	11·3		116		
Catalase (liver)								
Fluoride	$\lambda_{max.}$	600	None	(535)	~480	407	5·89	2, 4
	ϵ_{mM}	13·9		infl.	< 20	125		
Water pH 6·0		622	585	537	490	407	5·89	2, 4
		10·5	9·5	13·2	17	138		
Azide		620	580	530	490	412	5·86	2, 4
		12	10·3	12·8	16·8	138		
Cyanide		None	580	553	None	425	4·02	2, 4
			12·2	14·6		110		
Peroxidase (horseradish) (ferric)								
Fluoride	$\lambda_{max.}$	612	560	(530)	488	404	5·90	2, 4
	ϵ_{mM}	6·9	4·8	infl.	7·6	130		
Water pH 5·6		641	~580	~530	497	403	5·48	2, 4
		2·8	~2·5	~7·5	10·0	91		
Hydroxide		~635	572	545	None	416	2·66	2, 4
		~1·5	6·9	8·6		89		
Azide		635	565	534	(495)	416	—	2
		1·7	5·5	8·2	infl.	114		
Cyanide		None	568	538	None	423	2·67	2, 4
			7·5	10·2		94		

References: 1, Scheler, Schoffa & Jung (1957); 2, Keilin & Hartree (1951); 3, Scheler, Schoffa & Jung (1958); 4, Hartree (1946); 5, Theorell & Ehrenberg (1951).

Table 2. *The logarithm of the stability constants,* log K, *of some ferric complexes*

Ligand	H_2O	HCO_3^-	$CH_3CO_2^-$	F^-	OH^-	N_3^-
log K	0·0	3·1	3·2	5·2	11·5	4·5
Reference	—	1	1	2	2	2

References: 1, Perrin (1959); 2, Bjerrum, Schwarzenbach & Sillen (1958).

the character of the ligand, but the β-band intensity is almost independent of the fifth and sixth ligands. For the mainly low-spin ferric porphyrin complexes in Table 1 the intensity ratio α/β increases in the order cyanide < imidazole = ammonia < hydroxide. This is the order of increase of π-electron donor character of the ligands.

Rules for the identification of groups attached in ferric porphyrin complexes. This survey of the spectra of the ferric porphyrin complexes shows that three main correlations between spectra and magnetic moment exist.

(i) The intensity of the charge-transfer bands is high in high-spin complexes and very low (or zero) in low-spin complexes.

(ii) The intensity of the β-band is greater in low-spin complexes.

(iii) The Soret band is at longer wavelengths for low-spin as opposed to high-spin complexes. The change from high to low spin and the movement of the Soret band follows the ligand sequence fluoride > acetate = formate > hydroxide > cyanide for anions, and water > imidazole for neutral bases. There is also the possibility of obtaining information about the groups bound to the ferric ion in the fifth and sixth positions from the following correlations.

(iv) The charge-transfer bands move to shorter wavelengths the greater the electron density in the vicinity of the Fe^{3+} ion, owing to ligands in the fifth and sixth positions.

(v) The intensity ratio α/β increases the stronger the π-donor character of these ligands.

Before attempting to use these observations we will describe other properties of iron porphyrin complexes which are dependent upon the fifth and sixth ligands.

Ferrous complexes and redox potentials

A similar set of comparisons between absorption spectra and magnetic properties in the complexes of ferrous porphyrins, $Fe^{II}PXY$, has permitted diagnostic rules to be established.

(i) Strong π-acceptor ligands such as nitric oxide, cyanide, oxygen and carbon monoxide have a different effect upon the absorption spectra from all other ligands (Williams, 1956).

(ii) With the simple bases, such as ammonia, the intensity of the α-band is greater the stronger the π-donor character of the ligand.

(iii) The Soret band and the α- and β-bands move to shorter wavelengths the greater the percentage of low-spin form for all typical haemoproteins.

(iv) The α- and β-bands of purely low-spin ferrous porphyrin complexes move to longer wavelengths the stronger the donor character of the ligand.

(v) In the spectra of low-spin ferrous porphyrin complexes there is a band at about 500 mμ. This band also appears in simple low-spin ferrous complexes of unsaturated nitrogen bases, e.g. Fe^{II}(*o*-phenanthroline)$_3$.

(vi) The order of change of magnetic moment from high-spin to low-spin ferrous complexes is nitrogen bases > oxygen bases in all model complexes. Here oxygen base refers to a neutral group such as water or alcohol which acts as an electron donor through the oxygen atom.

The oxidation–reduction potentials of iron complexes can also be used diagnostically. The order of decreasing redox potentials of model complexes, Fe^{II}(dimethylglyoxime) (base)$_2$ as a function of the bases is pyridine > imidazole > water > ammonia > hydroxide (Williams, 1959*a*). (*N.B.* The imidazole group of a histidine residue of the protein can play the same role as imidazole in this connexion, and in general for the properties discussed in this paper.) There is no reason to suppose that the iron porphyrin complexes will differ in their behaviour from the iron dimethylglyoxime complexes with respect to the effect of the fifth and sixth ligands perpendicular to the porphyrin or dimethylglyoxime ring.

Diagnosis of co-ordination of iron porphyrin complexes

Adequate information on magnetic moments, spectra and oxidation–reduction potentials in the two oxidation states should allow us to predict the way in which the iron in a porphyrin complex is bound. For example, we can consider the series of proteins which have the same porphyrin group: catalase, peroxidase, myoglobin and haemoglobin. This is the order of increasing redox potential (Table 3). We are assuming here that the catalase potential is very low as it is not known to have been reduced to the ferrous state. The magnetic moment of these haemoproteins in the ferric state increases in the sequence peroxidase < methaemoglobin < metmyoglobin < catalase. If we con-

sider only the three ligands most probably available on the proteins (amino groups, imidazoles of histidine residues and carboxylate anions) and relate the above two sequences to our diagnostic rules on redox potentials and paramagnetism, we conclude that (*a*) haemoglobin and myoglobin are imidazole–water complexes with the imidazole more tightly (but still not very tightly) bound in haemoglobin, (*b*) peroxidase is an amino–carboxylate or less probably a water–amino complex, and that (*c*) catalase is a dicarboxylate or less probably a carboxylate–water complex.

This conclusion is substantiated in Table 3 where all the other diagnostic properties are considered. For each property the proteins are ordered according to the magnitude observed, as are, for comparison, model complexes with known pertinent ligands in the fifth and sixth positions. For example, the study of the intensity of the charge-transfer bands in the series of methaemoglobin complexes $Fe^{III}P$(histidine)Y, where the histidine is a residue of the protein, shows that the intensity falls in the sequence of ligands Y as follows: fluoride > carboxylate > water > hydroxide > imidazole = amines. This order is also observed for a series of haemin complexes (Scheler, 1960). Amongst the haemoproteins the order of intensity is catalase > methaemoglobin > metmyoglobin > peroxidase. We conclude that the groups which bind iron in catalase come earlier in the above sequence of ligands than those which bind iron in methaemoglobin and metmyoglobin. Similarly, the groups which bind to iron in peroxidase come later in the sequence. Table 3 shows that an account of all the properties of haemoproteins can be given if catalase is a carboxylate complex and peroxidase is an amino complex. Some chemical properties provide additional confirmation of this analysis.

Chemical properties of iron complexes

Ferric complexes. In Table 4 we list the pH at which certain ferric complexes are 50 % hydrolysed to give hydroxy compounds. It is particularly noticeable that complexes with zero or negative charge are difficult to hydrolyse. In general the higher the negative charge on the groups which bind the ferric ion the greater the pH of hydrolysis. Peroxidase, methaemoglobin and metmyoglobin hydrolyse at similar pH values. This suggests that the groups binding the iron carry the same or very similar numbers of negative charges. Thus, in the fifth position, the group binding iron in peroxidase is probably a nitrogen base. Catalase is not hydrolysed even in strong alkali. It seems likely that one if not both of the groups binding the iron in this haemoprotein is a carboxylate group.

Ferrous complexes. The model complexes Fe^{II}(dimethylglyoxime)$_2$(imidazole)$_2$ and Fe^{II}(dimethylglyoxime)$_2$(histidine)$_2$ can absorb both oxygen and carbon monoxide reversibly (Williams, 1959*b*). The complexes Fe^{II}(dimethylglyoxime)$_2$(amine)$_2$ ab-

Table 3. *Observed effects of ligands on the properties of model complexes compared with the observed properties of haemoproteins*

The model systems referred to are: A, iron dimethylglyoxime complexes; B, methaemoglobin with a variety of ligands in the sixth position, see Table 1; C, haemin complexes. Haemo, haemoglobin; Myo, myoglobin; Per, peroxidase; Cat, catalase; Imid, imidazole.

Property	Model	Order of property in model complex	Order of property in haemoproteins	Reference
Redox potential	A	Imid $\geqslant H_2O > NH_3 > OH^-$	Haemo > Myo > Per > Cat	1
Magnetic moment (ferric)	B	$NH_3 \leqslant$ Imid $< H_2O < CO_2^-$	Per < Haemo $\leqslant$ Myo < Cat	2, 3
Intensity of charge-transfer bands (ferric)	A, B, C	$CO_2^- > H_2O >$ Imid $\geqslant NH_3$	Cat > Haemo $\geqslant$ Myo > Per	1, 2, 4
Position of Soret band (ferric)	B, C	$NH_3 =$ Imid $> H_2O = CO_2^-$	Per $\simeq$ Haemo $\simeq$ Myo $\simeq$ Cat (very small differences)	2, 4
Position of charge-transfer bands (ferric)	B	$H_2O > CO_2^-$	Per > Myo = Haemo > Cat	2
Intensity ratio β:charge-transfer (ferric)	B, C	$NH_3 \geqslant$ Imid $> H_2O = CO_2^-$	Per > Haemo $\geqslant$ Myo > Cat	2, 4
Intensity ratio $\alpha:\beta$ (ferric)	B, C	$CO_2^- > NH_3 =$ Imid	Per < Haemo $\leqslant$ Myo < Cat	2, 4
Magnetic moment (ferrous)	A, B	Imid $= NH_3 > H_2O = CO_2^-$	Not known but spectra suggest Myo = Haemo > Per	1
Intensity ratio $\alpha:\beta$ (ferrous)	B	Imid $= NH_3 > H_2O$	Not known	1

References: 1, Williams (1959*b*); 2, Scheler, Schoffa & Jung (1957); 3, Hartree (1946); 4, Scheler (1960).

sorb carbon monoxide, but the absorption of oxygen which takes place in these complexes results in oxidation, i.e. is irreversible. Fe^{III}(dimethylglyoxime)$_2$(water)$_2$ does not absorb carbon monoxide to form Fe^{II}(dimethylglyoxime)$_2$(water) (carbon monoxide), and is immediately oxidized by oxygen. The iron porphyrin complexes Fe^{II}P-(pyridine)$_2$ absorb oxygen reversibly but only in the absence of water. (This evidence strongly suggests that oxygen is only absorbed reversibly if the atom bound to iron does not carry active hydrogen.)

Haemoglobin and myoglobin absorb oxygen and carbon monoxide reversibly. In these iron porphyrin complexes histidine is bound to the iron. Peroxidase with iron in the bivalent state absorbs carbon monoxide but is oxidized by molecular oxygen. This behaviour, in comparison with that of the model complexes above, indicates that the iron in peroxidase is not complexed with imidazole but is similar in its reactions with oxygen to an amine complex.

From the above analyses of physical and chemical properties it is clear that we can explain the characteristics of the four haemoproteins on the basis of differences in the groups binding the iron. Summarizing, we think of haemoglobin and myoglobin as FeP(histidine)(water), catalase as FeP-(carboxylate)$_2$ and peroxidase as FeP(amino)(carboxylate) or FeP(amino)(water).

Peroxide compounds of catalase, peroxidase, methaemoglobin and metmyoglobin

Catalase and peroxidase react with hydrogen peroxide and methyl and ethyl hydroperoxide to form four compounds, named I, II, III and IV according to the order of their appearance in the reactions. The reactions and the properties of the compounds formed have been reviewed by Keilin & Hartree (1951) and by Chance & Fergusson (1954).

Table 4. pH *at which hydroxide of ferric complex is* 50 % *formed*

Ligand	Charge on complex	pH of 50 % formation of hydroxide	Reference
Water $(H_2O)_6$	+3	2·5	1
(Phenanthroline)$_3$	+3	<2·0	2
(Dimethylglyoxime)$_2$	+1	4·5	3
Phthalocyanine	+1	5·5	4
8-Hydroxyquinoline	0	9·5	5
Ethylenediaminetetra-acetate	−1	7·5	5
Ethylenediaminetetra-acetate hydroxide	−2	>11·0	5
Methaemoglobin	—	8·9	6
Metmyoglobin	—	9·0	7
Peroxidase	—	~10·0	8
Catalase	—	No hydroxide formed	8

References: 1, Bjerrum, Schwarzenbach & Sillen (1958); 2, Gaines, Hammett & Walden (1936) (the hydroxy complex is the stable form even in strong acid); 3, Jillot & Williams (1958); 4, A. Riley & R. J. P. Williams (unpublished results); 5, Tomkinson & Williams (1958); 6, George & Hanania (1953); 7, George & Hanania (1952); 8, Keilin & Hartree (1951).

Table 5. *Spectroscopic and magnetic characteristics of peroxide compounds II and III of catalase and peroxidase, and of the peroxide compounds of methaemoglobin and metmyoglobin*

The band positions ($\lambda_{max.}$) are given in mμ and the millimolecular extinction coefficients (ϵ_{mM}) are per haematin. The magnetic moments are given in Bohr magnetons, β.

	α-Band		β-Band		γ-Band			
Compound	$\lambda_{max.}$	ϵ_{mM}	$\lambda_{max.}$	ϵ_{mM}	$\lambda_{max.}$	ϵ_{mM}	β	Reference
Catalase II	568	(13)	536	(12)	429	(70)	2·83	1, 2, 3
Catalase III	585	(11)	545	(12)	416	(84)	Unknown	3, 4
Peroxidase II	555	(7)	527	(8)	418	(87)	3·45	2, 3, 4
Peroxidase III	583	(8·5)	546	(10)	416	(97)	Unknown	3, 4
Peroxide compound of metmyoglobin	590	(8)	549	(9)	420	(100)	2·83	5, 6
Peroxide compound of methaemoglobin	585	(7·5)	545	(9)	Unknown		Unknown	3

References: 1, this paper (for experimental methods, see following paper); 2, Theorell & Ehrenberg (1952); 3, Keilin & Hartree (1951); 4, Chance (1952); 5, George & Irvine (1953); 6, Brill, Ehrenberg & Hartog (1960).

Methaemoglobin and metmyoglobin also react with these peroxides, and the resultant compounds have very much the same absorption spectrum as the compounds III of catalase and peroxidase. The optical and magnetic characteristics of compounds II and III of catalase and peroxidase and the peroxide compounds of methaemoglobin and metmyoglobin are given in Table 5, and these compounds will now be discussed. The compounds I, which are peculiar to catalase and peroxidase, we discuss in the next paper.

It has been suggested by George (1953) that compounds II of peroxidase and of catalase by analogy are quadrivalent (i.e. Fe^{IV}) iron complexes. More specifically he suggests that oxygen is bound in the sixth position so that compound II is a co-ordination complex of the hypothetical ferryl ion, $Fe^{IV}O^{2+}$. Peroxidase compound II has been shown by George to have one oxidizing equivalent above the parent haemoprotein, and its magnetic moment is that for two unpaired electrons with an orbital contribution. The magnetic moment of catalase compound II is exactly that for two unpaired electrons with no orbital moment. The only simple alternative to George's proposal is that compound II is a ferric complex and that the oxidizing equivalent is associated with the porphyrin ring as a stable free radical. The magnetic moment would then be made up of the radical's contribution plus that of the ferric ion which is then required to be in the low-spin state. Although the absence of the charge-transfer bands at 600–650 mμ and 450–500 mμ would be in agreement with this, the presence of the Soret band at its usual intensity argues against the oxidation of the porphyrin ring. It is also impossible to see how the β-bands of catalase and peroxidase compounds II, which are at 536 and 527 mμ respectively, can be at quite so short a wavelength. In their lowest-spin complexes, the cyanides, the β-band of catalase cyanide is at 553 mμ and that of peroxidase cyanide is at 538 mμ. In contrast with known ferric complexes of catalase and peroxidase, where the magnetic moments of the former always exceed those of the latter, the moment of catalase compound II is less than that of peroxidase compound II. These arguments are against the assumption that compound II is a ferric complex. There are several points which support George's postulation of ferryl iron, $Fe^{IV}O^{2+}$:

(i) Low-spin quadrivalent iron would have two unpaired electrons; the position of the Soret band, strongly displaced to the red, indicates a low-spin complex.

(ii) The intensity ratio α-band/β-band is greater than unity for catalase compound II and only a little less than unity for peroxidase compound II, positive evidence that a strong electron donor ligand, such as hydroxide or oxide, is bound to the iron.

(iii) The intensity ratio α-band/β-band is greater in catalase compound II than in peroxidase compound II. This ratio is generally greater in catalase complexes than in peroxidase complexes (Table 1). The α-band and β-band of catalase compound II are displaced about 11 mμ towards the red from the α- and β-bands of peroxidase compound II. Much the same difference in α- and β-band positions is found between other complexes of catalase and peroxidase (Table 1). Thus the compounds II exhibit the differences found between other complexes of catalase and peroxidase in which only the co-ordination in the sixth position is changed. This points to the preservation in compounds II of the co-ordination in the first five positions, and a common change in position six. This is not true of compounds III of catalase and peroxidase, however, which have very similar spectra. These spectra are very like those of the peroxide compounds of methaemoglobin and metmyoglobin, which suggests that one structure is common to them all, i.e. they all have the same co-ordination in the fifth and sixth positions. An obvious possibility is $Fe^{IV}P(hydroxide)_2$.

SUMMARY

1. A classification of the absorption bands of ferric porphyrin complexes is suggested. There are two groups of absorption bands, one group of two due to charge transfer to high-spin Fe^{3+} ions, and another group of three due to the absorption bands of the porphyrin. Only the second group of bands appears in low-spin complexes. An analysis of the absorption spectrum can be used to estimate the amounts of low-spin and high-spin ferric iron present in complexes which are equilibrium mixtures.

2. The relationships between the properties of known groups in the fifth and sixth co-ordination positions and the absorption spectra of iron porphyrin complexes are described. These relationships lead to a set of diagnostic rules by which the type of group bound to the iron porphyrin can be recognized. The rules for ferric and ferrous porphyrin derivatives differ and are separately discussed.

3. Magnetic, oxidation–reduction and some chemical properties provide additional evidence upon which the diagnosis of co-ordinating groups can be based.

4. As an illustration of the diagnostic method, the nature of the groups binding the iron in the haemoproteins catalase, peroxidase, haemoglobin and myoglobin is discussed, as is the co-ordination in the peroxide compounds II and III of catalase and peroxidase.

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Primary Compounds of Catalase and Peroxidase

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The mechanisms by which many haemoproteins fulfil their roles in biological oxidation and peroxide catalysis are not yet completely defined in spite of great experimental activity in this field of research. It would be of considerable help in arriving at an understanding of those mechanisms involving the higher oxidation states of haemoproteins if we knew the structures of the higher oxidation compounds. Great effort has been put into a determination of the absorption spectra (Keilin & Hartree, 1951; Chance, 1952*a*; George, 1953) of the higher oxidation compounds for it is only through the absorption spectra that these compounds can be followed separately and quantitatively through their reactions. Other properties, such as the magnetic moments and number of oxidizing equivalents of the compounds, can only be evaluated accurately after the absorption spectra have been established. The spectra themselves are useful in that they can be related to structure (see preceding paper).

Attention in this paper is directed particularly to the green primary compounds, 'compound I', of catalase and peroxidase with peroxides. Chance (1949*a*) has stated that alkyl hydroperoxides can convert free catalase completely into compound I. Our experiments show that this is only approximately correct, and we produce an absorption spectrum different from those arrived at in the past for primary compounds. The spectrum is not similar to any known haematin-containing protein, nor to any known porphyrin. We tentatively conclude that the oxidative event which has taken place is in part an attack on the porphyrin conjugated ring system, saturating it at one methene bridge. This conclusion is reinforced by similarities between our spectrum of compound I and the spectra of certain bile pigments.

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MATERIALS AND METHODS

Catalase was prepared at the Johnson Research Foundation, University of Pennsylvania, U.S.A., from *Micrococcus lysodeikticus* (American Type Culture Collection no. 4698) produced on a large scale in submerged culture (Beers, 1955). The enzyme was extracted by the method of Herbert & Pinsent (1948) and purified by repeated precipitations with acetone from neutral 0·01 M-phosphate buffer (A. S. Brill & A. C. Maehly, unpublished work). Catalase concentration was measured by light-absorption at 406 mμ (Soret peak) in a Unicam quartz spectrophotometer. The millimolar extinction coefficient (E_{mM}) of the Soret peak is 103 mM^{-1} cm.$^{-1}$ per haematin group. The Kat. f. (Euler & Josephson, 1927) for this preparation is 143 000 at 25° as determined by the method of Bonnichsen, Chance & Theorell (1947).

The maximum purity value obtainable for bacterial catalase is 0·92 where purity value is defined as the ratio $E_{406\ m\mu}/E_{280\ m\mu}$. The purity value of the enzyme solutions used in these experiments was in the range 0·86–0·88 except for one set of measurements in the visible for which it was 0·82. Low absorption in the u.v. is necessary for the success of the experiments to be described below.

In preparation for each set of experiments, a concentrated solution of enzyme was dialysed against 0·01 M-phosphate buffer, pH 7·25±0·05. All dilutions were made with this type of buffer.

A Cary recording spectrophotometer was used. Preceding each set of experiments a base-line was obtained over the spectral region of interest by recording with water in both the sample and reference cuvettes. Then the diluted catalase solution was placed in the sample cuvette, and the absorption spectrum of the free enzyme was recorded as a check upon the concentration and upon the proper functioning of the spectrophotometer.

Ethyl hydroperoxide was most kindly given by the Laboratory of Laporte Chemicals Ltd. Its concentration was measured by light-absorption at 230 mμ, where E_{mM} is 0·043 mM^{-1} cm.$^{-1}$ (Reiche, 1931).

The temperature at which these experiments were performed was not controlled, and varied from 24 to 26°.

In the paramagnetic resonance experiments a radio-frequency field-modulated spectrometer of the type described by Llewellyn (1957) was employed. The magnetic field was slowly swept through the region of interest, a band width of 2 cyc./sec. was employed, and the signal was traced with a recording milliammeter. The sensitivity was calibrated before each experiment with the same crystal of the Tutton salt, $Zn(NH_4)_2(SO_4)_2,6H_2O$, containing 1·6% of $Cu(NH_4)_2(SO_4)_2,6H_2O$. In this salt the bivalent ions are in two differently orientated complexes, there being about $1{\cdot}3 \times 10^{15}$ Cu^{2+} ions of each type in the calibrating crystal. The signal-to-noise ratio at 90° K for a hyperfine line of half-width 7 oersteds was eight.

RESULTS

Conditions for maximum formation of compound I

When ethyl (or methyl) hydroperoxide reacts with catalase a transient green compound is formed, compound I. As compound I disappears, compound II and free catalase appear. (A summary of the peroxide compounds of catalase and peroxidase is presented in the preceding paper.) The experiment described in this section demonstrates that the maxima of concentration of compound I reached in this reaction are a non-equilibrium function of the concentrations of peroxide and enzyme.

The wavelength was fixed at 405 mμ, and the extinction of free catalase solution was recorded. Upon a signal, between 0·05 and 0·10 ml. of ethyl hydroperoxide solution was blown from a pipette into the cuvette containing 3·0 ml. of enzyme solution, and the reaction mixture stirred with a rod previously placed in the cuvette. The time which elapsed from the signal until the stirring rod was removed was 7 or 8 sec. The recording was started exactly 10 sec. after the signal. Fig. 1 shows one such recording.

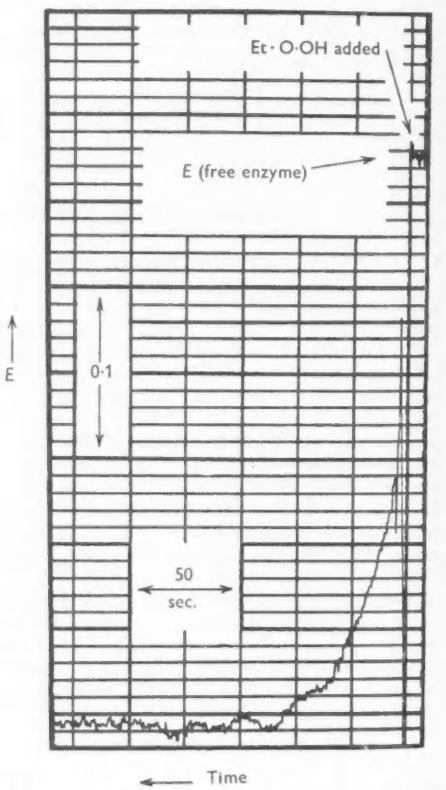

Fig. 1. Recording of $E_{405\ m\mu}$ showing steady state. Final concentrations: 6·32 μM-catalase-haematin, 295 μM-ethyl hydroperoxide, 0·01 M-phosphate; pH 7·25.

Depending upon the concentration of peroxide, a steady state is reached in 35–80 sec. and lasts 15–20 sec. before E begins to change again. The change in E from that of free catalase to that of the steady-state reaction mixture is a measure of the amount of free enzyme converted into compound I. Table 1 shows the relationship between peroxide concentration and compound I formation. The latter is maximal at a peroxide concentration of about 300 μM.

Absorption spectrum of the steady-state reaction mixture

Under conditions (300 μM-ethyl hydroperoxide) of maximum conversion into compound I, the experimental routine described in the previous section was performed at 15 mμ intervals from 300 to 435 mμ and from 510 to 690 mμ, and also at 460 and 485 mμ. The concentrations of enzyme for the experiments in the Soret and u.v. regions were 6–8 times less than that used in the visible. The difference in E between the free enzyme solution and the steady-state reaction mixture was divided by the enzyme concentration to obtain ΔE_{mM}. The enzyme concentration used in this calculation was the average of that before and after adding the ethyl hydroperoxide (which differed at most by 3·2%). Values of ΔE_{mM} were then subtracted from the extinction coefficients for the free enzyme and plotted as curve A, Fig. 2.

Because the reaction mixture is almost entirely compound I for a period of about 2 min. during and after the steady-state period, it was possible to use the wavelength-scanning properties of the Cary spectrophotometer to assist in filling in the regions between the points determined from the steady-state measurements. Fig. 3A shows a scanning trace started at the beginning of a steady state at 550 mμ and run during 6·25 min. up to 700 mμ. Fig. 3B is a trace started about 20 min. after the start of the trace in Fig. 3A and shows that compound II is now the major component of the reaction mixture.

Table 1. *Concentration of compound I as a function of ethyl hydroperoxide concentration*

pH 7·25; 0·01 M-phosphate; 25°; spectrophotometric measurements at 405 mμ with 1 cm. optical depth.

Catalase-haematin (μM)	Ethyl hydroperoxide (μM)	Difference in E/catalase-haematin (mM^{-1})
5·04	32	−30·5
5·08	98	−49·3
5·25	286	−54·3
6·36	295	−53·0
6·32	560	−48·0

Change in extinction on adding cyanide to a steady-state reaction mixture

Chance & Herbert (1950) have shown that cyanide combines rapidly with bacterial catalase, and have used this fact to demonstrate that,

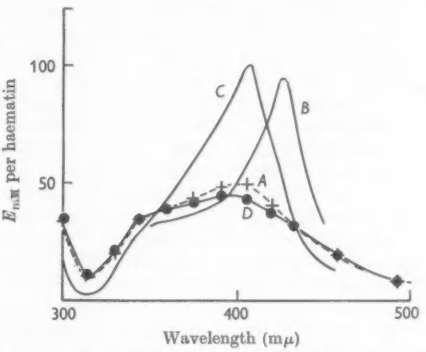

Fig. 2. Absorption spectra of A, the steady-state reaction mixture of catalase (6·3 μM-haematin) and ethyl hydroperoxide (300 μM); B, the cyanide complex (4·1 μM-haematin, 360 μM-potassium cyanide); C, free catalase. 0·01 M-Phosphate, pH 7·25, for all curves. The crosses on curve A indicate those wavelengths at which changes in extinction coefficient were determined from steady-state measurements. D is the calculated curve for compound I.

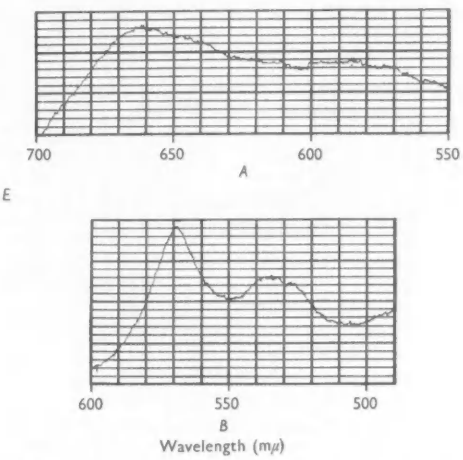

Fig. 3. A, Recording of E scanning from 550 mμ at the beginning of the steady state to 700 mμ 6·25 min. later. B, Recording of E scanning from 490 to 600 mμ, starting about 20 min. after the steady state was reached. Final concentrations, A and B: 32 μM-catalase-haematin, 610 μM-ethyl hydroperoxide, 0·01 M-phosphate, pH 7·25.

during the catalatic decomposition of hydrogen peroxide, substantially more than half of the catalase haematins are free. The Soret spectrum of the cyanide complex of their enzyme differs negligibly from ours (Fig. 2*B*) in the location and extinction coefficient of the peak at 426 mμ, but absorbs substantially more light below 410 mμ.

Chance (1949*a*) added cyanide to a mixture of horse-liver catalase and ethyl hydroperoxide and recorded the change of $E_{435\ m\mu}$. Finding 'no appreciable' rapid reaction with cyanide, he concluded that ethyl and methyl hydroperoxide can bind all three of the haematins of horse-liver catalase. The difficulty in detecting the presence of free catalase at 435 mμ is the same as that at 426 mμ which is described in the last paragraph of this section. Without presenting further evidence, Chance & Herbert (1950) assumed that methyl hydroperoxide binds completely all four haematins of bacterial catalase. We show that free catalase is still present under the best conditions for the formation of compound I from ethyl hydroperoxide. From the absorption spectrum given by Chance & Herbert, we infer that free catalase is also present in the reaction mixture with methyl hydroperoxide.

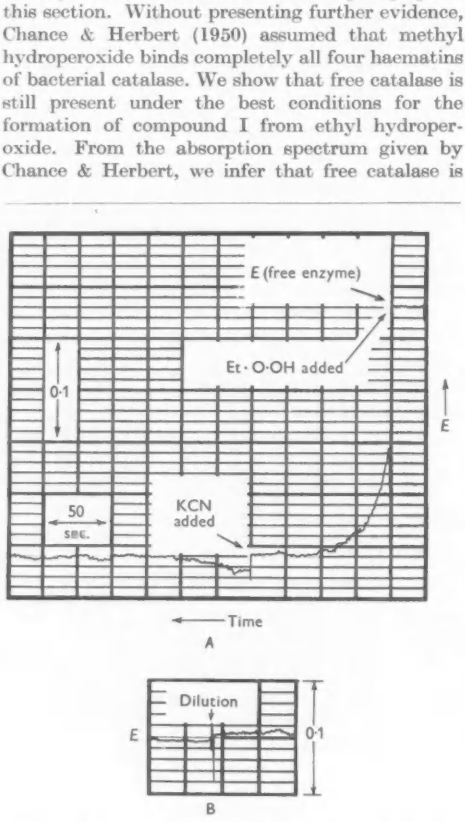

Fig. 4. *A*, Recording of *E*, showing effect of addition of cyanide (0·085 ml. of 0·013 M-potassium cyanide) to the steady-state reaction mixture (3·00 ml. of 4·2 μM-catalase-haematin, 0·052 ml. of 0·017 M-ethyl hydroperoxide). 0·01 M-Phosphate, pH 7·25, λ 405 mμ. *B*, Recording of *E*, showing the dilution effect; λ 405 mμ.

At 405 mμ the value of ΔE_{1mM} for the change from free catalase to the cyanide complex is -49 mM^{-1} cm.$^{-1}$ per haematin. Indeed, as can be seen in Fig. 2, the absorption of the cyanide complex at 405 mμ is only slightly greater than that of the steady-state reaction mixture. If cyanide is added to the steady-state reaction mixture while the spectrophotometer is set at 405 mμ, *E* will drop quickly, if free catalase is present, owing to formation of the cyanide complex. *E* can drop only if free enzyme is present before addition of cyanide since conversions from compound I and compound II into the cyanide complex are accompanied by increases in *E*.

A steady-state reaction mixture was produced as described in the preceding sections. When the steady state was reached, the recorder was stopped, and 0·085 ml. of 0·013 M-cyanide solution was blown into the reaction mixture containing 3·00 ml. of 1·06 μM-catalase (4·24 μM-haematin) solution and 0·052 ml. of 0·017 M-ethyl hydroperoxide. The recorder was started 10 sec. later. Fig. 4*A* is a record of one such experiment. The drop in *E* upon the addition of cyanide is apparent. However, part of this drop is due to dilution. Fig. 4*B* shows the dilution effect in an optically identical but non-reacting solution, 0·0055 extinction unit, which must be subtracted from the change observed in Fig. 4*A*. There remains a decrease of over 0·01 extinction unit, which demonstrates unmistakably the presence of free haematin groups.

The conversion of compound I back into free catalase, which is immediately complexed by the cyanide, can be seen at the end of the record of Fig. 4*A*. This conversion can be studied more readily at 426 mμ where the difference in light-absorption between compound I and the cyanide complex is greatest. The conversion, as shown in Fig. 5, is accurately first-order with a rate constant of $8{\cdot}6 \times 10^{-3}$ sec.$^{-1}$. Therefore when the spectrophotometer was started 10 sec. after the cyanide was introduced, 8% of the compound I which was present had already been converted into the cyanide complex. In the record shown in Fig. 4*A*, for example, the drop in *E* must be increased by 0·002. All factors taken into account, the amount of free catalase present at the steady state is at least 5% but not more than 10% of the total catalase. It should be possible to apply a rapid-flow technique to this method and achieve great accuracy in establishing the composition of the steady-state reaction mixture.

Fig. 5 shows that at 426 mμ it is difficult to determine the unbound catalase accurately. Because the changes in *E* from free catalase and

from compound I to the cyanide complex have the same sign, the immediate and exponential changes are in the same direction. The later change is rapid and would have to be accurately extrapolated to zero time. However, when dilution is considered, the effect of the free catalase is apparent.

Paramagnetic resonance experiments

The growth of crystals of bacterial catalase of sufficient size for solid-state paramagnetic resonance measurements has thus far been unsuccessful. We have made measurements on solutions where the broadness of the lines of the iron makes their detection impossible, but where the presence of free radicals might be observed. In earlier paramagnetic resonance measurements on aqueous solutions and freeze-dried samples, compound I of peroxidase has not produced a free radical signal (Chance & Fergusson, 1954).

Two factors determine the concentration of free radicals which can be detected, the width of the absorption band and the saturation of the spin system. The spin-lattice relaxation times of free radicals are frequently long and saturation may occur at moderately high levels of input power. However, the intramolecular magnetic interactions among possible free radicals and the four iron atoms of catalase produce a short spin–spin relaxation time and saturation should not be reached in this particular spin system at the power used. Indeed, the intramolecular dipolar fields in haemoproteins seriously decrease the possibility of observing a signal from free radicals within the molecule because of line-broadening. A free radical in a porphyrin ring would be in the field of an iron atom distant only a few Ångström units. The line width of this radical signal would be too great for the absorption to be detectable. (The frequency of the Brownian rotation of the catalase molecule is low compared with the frequency corresponding to the line width. Whence the orientation of the intramolecular fields with respect to the applied d.c. field are not averaged over a sufficiently short time to decrease the effect of the dipolar interactions.) However, a radical in the protein moiety might be observed. The field at a radical, due to an iron atom of unit spin at a distance of 10 Å, would produce a line-broadening of (half) width less than 30 gauss (G). This signal would be observable.

Samples of steady-state reaction mixture (0·1 ml., 65–70 μM-iron, 4×10^{15} spins) were introduced into small Pyrex tubes which were then placed in the 3 cm. cavity of the spectrometer. At room temperature the region g (Landé splitting factor) = 1·9–2·3 was examined in sections appropriate to the lifetime of the green catalase compound. The absorption was the same as with the Pyrex tube alone. Assuming a line of width (half) of about 30 G for possible free radicals in the protein moiety, their concentration was less than 20 % of that of the iron. In experiments in which the reaction mixture was quickly brought to liquid-oxygen temperature, again no resonance was detected; this, for the 30 G line width, indicates that the concentration of free radicals was less than 4 % of that of the iron. The absence of a signal in the $g = 2$ region suggests that the protein moiety of compound I does not contain a free radical. [There is evidence (Brill, 1960) that free-radical intermediates are produced when reducible substances are present.]

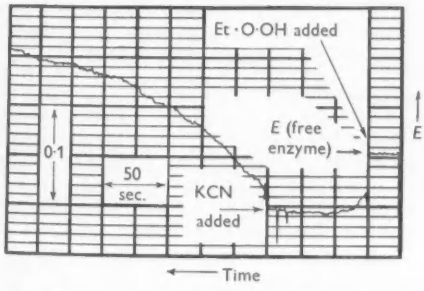

Fig. 5. Recording of E, showing the effect of addition of cyanide (0·085 ml. of 2·62 mM-potassium cyanide) to the steady-state reaction mixture (3·00 ml. of 4·2 μM-catalase-haematin; 0·052 ml. of 0·017 M-ethyl hydroperoxide). 0·01 M-Phosphate, pH 7·25, λ 426 mμ.

DISCUSSION

Under the conditions in which potassium cyanide was added to the preformed steady state of catalase and ethyl hydroperoxide a minimum of 5 % of the catalase was in the free state whereas a minimum of 90 % was not free. We have calculated the 'best' spectrum for compound I assuming that the steady state contains 92·5 % of compound I (Figs. 6, 7). It is not possible on the basis of our experiments to define the steady state more closely than this. The spectrum of compound I of bacterial catalase is not finally established therefore, but several new features (ii–v) appear in the following list of characteristics: (i) There is a peak at 662 mμ. (ii) There is a broad absorption band from 570 to 600 mμ with a maximum at 585 mμ but not distinct α- and β-bands. (iii) There is increased absorption at 450 mμ over that observed in free catalase. (iv) The intensity of the Soret peak is considerably less than half the free-catalase value and very low compared with all other catalase complexes; its position is obscure. (v) There is increased absorption in the region 290–360 mμ over free catalase.

We have looked at the spectrum of peroxidase compound I, which is well established (Fig. 8), carefully seeking similarities with the catalase compound I spectrum. We note the following points. We have preferred the spectrum given by George (1953) for compound I in the visible and that of Fergusson (1956) in the ultraviolet for these authors give the most detailed spectra in the respective regions. Where comparisons can be made, there is little disagreement among different authors (i.e. Keilin & Hartree, 1951; Chance, 1952*a*). We have not distinguished here between the different methods of preparing compound I, either from H_2O_2 or from reagents such as K_2IrCl_6. The reasons for not making any distinction are largely those of Chance (1952*b*) and Fergusson & Chance (1955). They will become clear in our discussion. (i) There is a band in the region of 650 mμ which is weaker than the 662 mμ band of catalase. (ii) There is a relatively much stronger broad band at about 540–590 mμ. (iii) There is an absorption band in the region of 420–490 mμ. The absorption in the region 420–450 mμ is higher than that of free peroxidase. (iv) There is a weak Soret band but it is more pronounced than in catalase, being a little more than half of the usual intensity of the Soret bands in peroxidase complexes. This much weakened Soret band of compound I is at a longer wavelength than that of peroxidase itself. (v) There is quite high absorption in the region of 360 mμ, but the absorption here is relatively less than in catalase compound I. There is no evidence as yet that peroxidase compound I absorbs more strongly than does peroxidase itself at shorter wavelengths than 360 mμ, although this looks possible from the spectrum of Fig. 8.

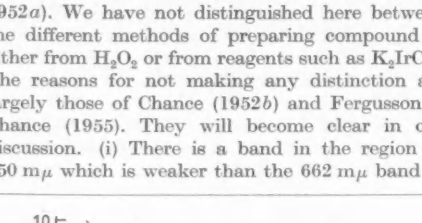

Fig. 6. Absorption spectrum of catalase compound I in the visible region, corrected for the presence of 7·5% of free catalase in the steady-state reaction mixture. Points indicate the wavelengths at which changes in extinction coefficient were determined from steady-state measurements. The spectrum of free catalase is given for comparison.

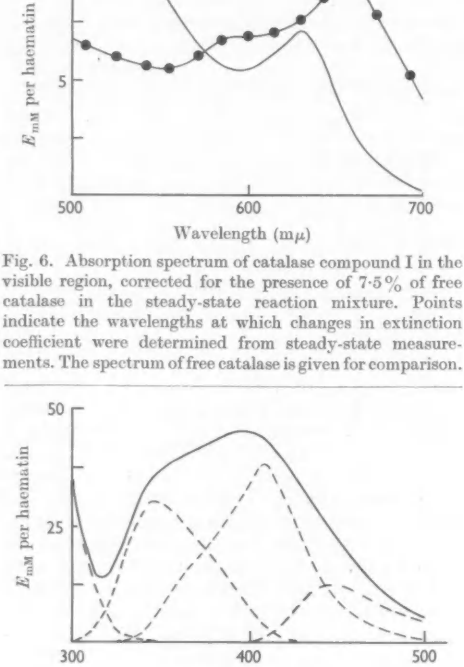

Fig. 7. Solid curve: absorption spectrum of catalase compound I in the u.v. region, corrected for the presence of 7·5% of free catalase in the steady-state reaction mixture. Broken curves: analysis of this spectrum into three absorption bands.

Before attempting to relate the spectra of the two compounds I, we draw attention to the following further facts about the catalase spectrum. The absorption in the region 330–500 mμ is obviously not due to a single peak. It can be formulated most simply as a combination of three absorption peaks with maxima at 340–360, 405–415 and 450–460 mμ (Fig. 7). The ratios of the heights of the assumed absorption peaks at 360 and 405 mμ to that at 662 mμ are about 3:1 and 4:1 respectively. The peak-height ratio 405 mμ:662 mμ is very low compared with the corresponding ratio (usually 8–15:1) of peak heights of the Soret band to any band in the visible region of the spectra of other iron protoporphyrin complexes. On the basis of these observations we regard the spectrum of compound I as being composed of two parts.

(*a*) A part due to a normal porphyrin-type complex with absorption in the Soret region 400–410 mμ, and in the visible between 440 and

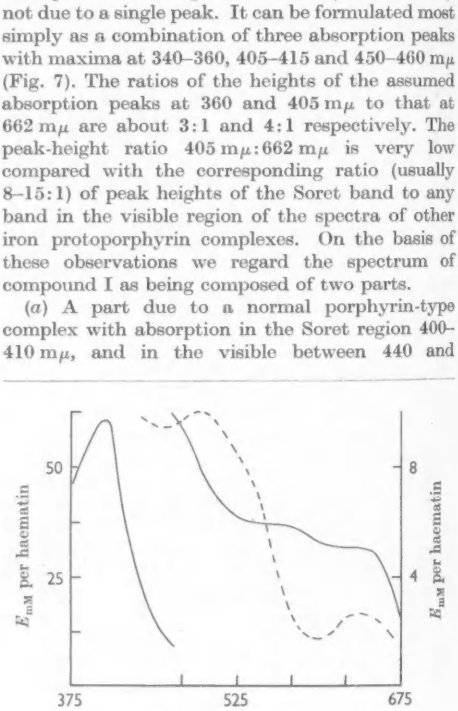

Fig. 8. Absorption spectrum of compound I of peroxidase. Soret-band region is from Fergusson (1956). Visible region is from George (1953). The spectrum of free peroxidase is given for comparison (broken curve).

480 mμ (the second charge-transfer band, see preceding paper) and between 540 and 600 mμ (the α- and β-bands).

(*b*) A part due to some other type of complex which has bands in the region 340–360 mμ and at 662 mμ.

In clarification of point (*b*) we note that no known ferric protoporphyrin complex has such strong bands as low as 360 mμ or as high as 662mμ. The usual δ-bands of haemoproteins are only about one-fifth of the intensity of the Soret bands. We have also examined the published spectra of compound I of erythrocyte catalase (Chance, 1952*a*) and find that, although undoubtedly partly in error, it has most of the outstanding features mentioned above.

This analysis of the spectrum of compound I of catalase can be extended to compound I of peroxidase, and shows that there is a close similarity between the two. We describe each as a combination of the two component spectra. For catalase compound I it is spectrum *b* with bands at 360 and 662 mμ which predominates, whereas for peroxidase it is spectrum *a* with bands at 410, 480 and 580 mμ which predominates.

Magnetic-susceptibility measurements on the reaction of bacterial catalase with methyl hydroperoxide (Brill, 1960) show a change in molar susceptibility on going from the free enzyme to the steady-state reaction mixture of -7800×10^{-6} e.m.u. at 20°. In order to calculate the concentration of compound I in the reaction mixture we need to know its spectrum. Rather than presume that the spectrum of catalase methyl hydroperoxide compound I is the same as that of ethyl hydroperoxide compound I, we have used the published data of Chance & Herbert (1950). We believe this spectrum to be incorrect, owing to the presence of about 10% of free catalase and the magnetic moment we calculate for catalase compound I is probably too high. Free catalase is a high-spin ferric complex of molar paramagnetic susceptibility 14 000–14 500 $\times10^{-6}$ e.m.u., which makes the susceptibility of compound I 6000–6500 $\times10^{-6}$ e.m.u. Theorell & Ehrenberg (1952) obtained a value of 6500×10^{-6} e.m.u. for compound I of peroxidase. These susceptibility values are close to the 'spin only' value of 6340×10^{-6} e.m.u. for a complex with average spin 3/2.

Thus the two compounds I of peroxidase and catalase are similar in magnetic moment. Table 2 shows that in those complexes where catalase and peroxidase have intermediate moments corresponding to between 1 and 5 unpaired electrons, the peroxidase complex has much the lower moment. This suggests that compound I is unlikely to be a simple complex of ferric iron which is the same for both catalase and peroxidase.

The magnetic evidence allows the possibilities that compound I is (*a*) a simple Fe^{V} complex of spin 3/2, (*b*) a mixture of iron complexes of average spin per iron close to 3/2, (*c*) a radical in the (i) porphyrin or (ii) protein moiety combined with a strong field Fe^{IV} state (unit spin), (*d*) a diradical in the porphyrin (unit spin) combined with a strong field Fe^{III} state (spin 1/2). The long half-life of compound I argues against the assumption of any radical, and we therefore are inclined to dismiss (*c*) and (*d*). Additional evidence against (*c* ii) is provided by the paramagnetic-resonance absorption spectra of catalase and peroxidase compounds I which give no signal for a radical of $g=2$. We are unable to offer an explanation of the spectra of the peroxidase and catalase complexes on the basis of assumption (*a*). No metal porphyrin complex of spin 3/2 which is not a mixture of spin states is known. However, the Cr^{III} phthalocyanine complexes (spin 3/2) have been studied (Anderson, Bradbrook, Cook & Linstead, 1938). Their spectra are in no way exceptional. Phthalocyanines and porphyrins resemble one another closely in spectroscopic properties, and we would expect Fe^{V} porphyrin complexes to have a typical metal porphyrin spectrum. The spectrum of compound I is very different from this. We can offer the following explanation of the spectrum on assumption (*b*) that compound I is a mixture of two components. We shall attempt to show that they are the ferric complexes given in Fig. 9.

Component *A*, which we shall call 'POR', appears to retain the porphyrin ring intact. POR could be a simple ferric porphyrin complex, either

$$Fe^{III} \leftarrow HO_2Et \text{ or } Fe^{III}O_2\text{–}Et$$

with a proton attached elsewhere to the protein. It is highly probable that the initial step in the peroxide reaction with peroxidase (or catalase) is the direct attack of the peroxide on the iron atom. The evidence is that peroxidase is oxidized to compound I by peroxides at pH 7·0, but not at pH 11·0 (Chance, 1949*b*), when peroxidase is present as the Fe^{III}–OH^- complex. The latter is a

Table 2. *Magnetic moments in Bohr magnetons of complexes of catalase and of peroxidase* (*Hartree*, 1946)

	F^-	H_2O*	N_3^-	SH^-	CN^-	OH^-
Catalase	5·89	5·89	5·86	4·20	4·02	—
Peroxidase	5·90	5·48	—	2·40	2·67	2·66

* The H_2O column refers to catalase and peroxidase themselves.

strong-field complex (low-spin) which exchanges ligands slowly and cannot form a complex $Fe^{III} \leftarrow HO_2Et$ rapidly. Catalase, which undergoes no corresponding acid dissociation, reacts with peroxides independently of pH (Chance, 1952*c*). On the other hand, K_2IrCl_6 and $K_3Mo(CN)_8$ oxidize peroxidase to compound I only in alkaline solution (George, 1953). Strong-field complexes are more readily oxidized by electron-transfer reagents such as K_2IrCl_6 and $K_3Mo(CN)_8$. The reaction with chloroiridate would proceed as in Fig. 10. The final complex is identical with the component *B* of

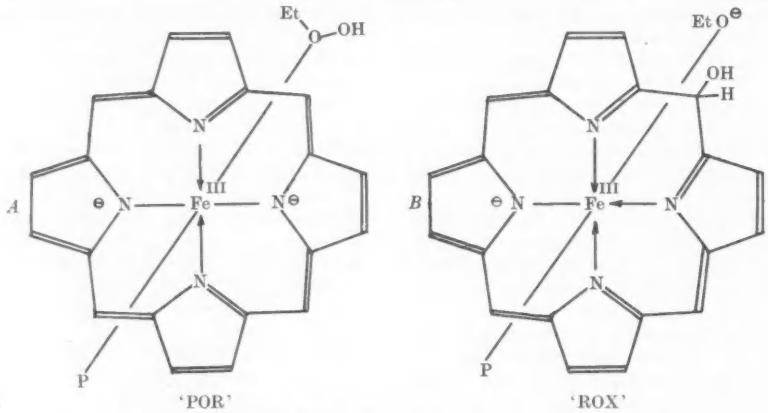

Fig. 9. The suggested components (*A* and *B*) of compound I. P, Protein.

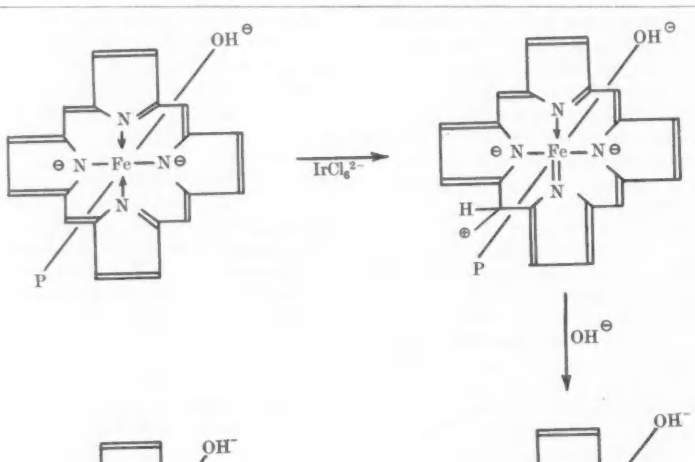

Fig. 10. Proposed reaction of peroxidase with chloroiridate in alkaline solution. P, Protein.

Table 3. *Properties of the primary peroxide compounds*

The approximate fractions of enzyme present at equilibrium as the ROX and POR components have been estimated from the spectra of the compounds I. The magnetic moments of POR of catalase and ROX of peroxidase have been taken as 5·9 and 2·1 Bohr magnetons respectively, and the moments of ROX of catalase and POR of peroxidase computed from $\mu^2_{meas.} = f\mu^2_{ROX} + (1-f)\mu^2_{POR}$, where f is the estimated fraction of enzyme present as ROX and $\mu_{meas.}$ is the measured magnetic moment.

		Absorption maxima (mμ)	Approx. fraction	Magnetic moment (β)
Catalase	ROX	360, 662	2/3	2·3
	POR	405, 450, 585	1/3	5·9
Peroxidase	ROX	360, 650	1/3	2·1
	POR	410, 480, 580	2/3	4·5

compound I (Fig. 9) which we expect to be formed by H_2O_2. It is also a tautomer of component *A*. The proportion of *A* to *B* will be a function of the relative stability of these components.

Component *B*, which we shall call 'ROX' (for oxidized ring), is not a porphyrin but a bile-pigment type of compound. It has a system of conjugated bonds like that of glaucobilin (Stern & Pruckner, 1937), or hydroxyporphyrin haematin (Lemberg, Cortis-Jones & Norrie, 1938). Glaucobilin has absorption bands at 360 mμ and 650–680 mμ and, unlike porphyrins, the ratio of the intensities of the bands is roughly 3·0. Hydroxyporphyrin haematin also has a spectrum with bands at 360 mμ and above 600 mμ. These spectra bear marked similarities to that of part *B* of the compound I spectrum.

The magnetic moment of compound I demands that this ferric complex (assuming that it is a ferric complex) should have an average spin of about 3/2. We expect ROX, as a ferric–hydroxyl type of complex to have a moment less than that for spin 5/2 and probably closer to spin 1/2. POR is probably also of spin less than 5/2, but certainly of greater spin than ROX. The moments of the POR and ROX components of peroxidase should be lower than those of catalase to be consistent with known complexes (Table 2). The order of magnetic moments is very likely

$$POR_{cat.} > POR_{per.} > ROX_{cat.} > ROX_{per.}$$

so that the average moment of compound I of peroxidase (which is mostly POR) can well be the same as that of compound I of catalase (which is mostly ROX).

The changes in the absorption spectrum of ferric haemoproteins which occur as they are changed from the high-spin (5/2) type to types of intermediate spin (between 5/2 and 1/2) such as POR are known from studies of, for example, the influence of pH changes on the spectrum and paramagnetic susceptibility of metmyoglobin (Theorell & Ehrenberg, 1951). As the magnetic moment falls we expect (*a*) a movement of the Soret band to longer wavelengths, (*b*) increased absorption between 530 and 590 mμ, and (*c*) decrease in absorption at 630 mμ, and between 500 and 530 mμ. The same correlation, discussed in detail in the preceding paper, between changes in the absorption spectrum and magnetic moment has been shown by varying the ligand in methaemoglobin complexes (Scheler, Schoffa & Jung, 1957). All these changes but one occur in the peroxidase reactions with hydroperoxides and are due to POR. The exception is the increase in absorption at 630 mμ which we have already attributed to the presence of some of the oxidized ring structure, ROX. For catalase, the Soret band does not shift perceptibly to the red because the POR component of catalase compound I should be of quite high spin. Also ROX is the major component and has a greater influence on the spectrum, tending to shift the Soret band to shorter wavelength.

Table 3 summarizes our views about the nature of compound I. It will be seen that the two molecules suggested as components of compound I are such that migration of a group changes one to the other. If this is so it is pointless to discuss whether there is an enzyme–substrate complex or a chemical compound. Our theory is open to test for it requires that the ferric ion in complexes like ROX should have a low spin.

SUMMARY

1. A study has been made of the properties of the primary compound formed between ethyl hydroperoxide and bacterial catalase. The compound is incompletely formed under the experimental conditions and its physical properties have been deduced from an examination of the steady-state reaction mixture.

2. The absorption spectrum obtained for compound I differs from that reported in the literature. It has a very weak Soret band, a band at approximately 360 mμ and two bands in the visible at approximately 580 and 660 mμ. This spectrum is unlike that of any other iron porphyrin complex.

3. Consideration of the physical and chemical properties of compounds I of bacterial catalase and of peroxidase has led us to suppose that these compounds are mixtures of two components. One of the components is a simple ferric porphyrin complex with the hydroperoxide. The other is the result of an attack of the hydroperoxide upon the porphyrin ring.

The authors wish to thank Professor Sir Hans Krebs, F.R.S., for providing laboratory facilities for preparing the solutions and for the use of the Cary spectrophotometer.

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Studies in Detoxication

85. THE METABOLISM OF *m*-DINITRO[^{14}C]BENZENE IN THE RABBIT*

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(*Received* 25 *July* 1960)

m-Dinitrobenzene is used industrially as a dyestuff intermediate and as a constituent of mining explosives. Animals poisoned with *m*-dinitrobenzene develop methaemoglobinaemia, anaemia, liver damage, convulsions and cerebral paralysis (Kunz, 1942; Kiese, 1949). The tissues of rabbits dosed with *m*-dinitrobenzene have been shown to contain *m*-nitroaniline (Belaborodova, 1945) and *m*-nitrophenylhydroxylamine (Lipschitz, 1920), and reduction of *m*-dinitrobenzene to *m*-nitrophenylhydroxylamine has been demonstrated *in vitro* in rabbit muscle (Comel, 1931) and blood (Lipschitz, 1948). In the presence of fermenting yeast, *m*-dinitrobenzene is converted into *m*-nitroaniline and 3:3′-dinitroazoxybenzene (Neuberg & Reinfurth, 1923) which is probably an artifact of *m*-nitrophenylhydroxylamine formed by atmospheric oxidation. The formation of dinitroazoxybenzene is evidence of the probable stepwise reduction of *m*-dinitrobenzene to *m*-nitrosonitrobenzene, *m*-nitrophenylhydroxylamine and *m*-nitroaniline.

The present work with *m*-dinitro[^{14}C]benzene has made it possible to account for the greater part of an oral dose, most of which is quickly excreted in the urine as *m*-nitroaniline, *m*-phenylenediamine, 2-amino-4-nitrophenol and 2:4-diaminophenol, together with traces of 2:4-dinitrophenol, 4-amino-2-nitrophenol, *m*-nitrophenylhydroxylamine and artifacts derived from this, and unchanged *m*-dinitrobenzene.

EXPERIMENTAL

Melting points are corrected.

Preparation of m-*dinitro*[^{14}C]*benzene.* *m*-Dinitro[^{14}C]benzene, m.p. 91°, randomly labelled with ^{14}C in one carbon atom, was obtained as a by-product of the preparation of nitro[^{14}C]benzene (Parke, 1956). Unlabelled *m*-dinitrobenzene (2 g.) was added to the solid residue which remained after distillation of the nitro[^{14}C]benzene, and the material was recrystallized from ethanol until it was shown

* Part 84: Parke (1960).

to be free (< 0·1%) from *o*-dinitrobenzene, the principal radioactive impurity, by the colour test described by Fearon & Kawerau (1943). *m*-Dinitrobenzene (10 mg.) in 0·5 ml. of ethanol was heated with 1 ml. of aq. ascorbic acid solution (0·1%, w/v) and 0·2 ml. of 2N-NaOH in a boiling-water bath for 30 sec. The mixture was diluted to 10 ml. with water and the light-absorption at 560 mμ was measured within 5 min. The violet colour that *o*-dinitrobenzene yields under these conditions has $\lambda_{max.}$ 560 mμ (ϵ, $2{\cdot}4 \times 10^6$). By this means 10 μg. of *o*-dinitrobenzene can be detected in 10 mg. of *m*-dinitrobenzene.

Materials. The following were used: *m*-nitroaniline, m.p. 114°, and 3-nitrobenzanilide, m.p. 159°; *m*-phenylenediamine, m.p. 64°, and its *NN'*-dibenzoyl derivative, m.p. 240°; 2:4-dinitrophenol, m.p. 113°, and its toluene-*p*-sulphonate, m.p. 122°; 3:5-dinitrophenol, m.p. 126° (Sidgwick & Taylor, 1922); 2:6-dinitrophenol, m.p. 61°; 2:4-diaminophenol, m.p. 80°, and its *ONN'*-tribenzoyl derivative, m.p. 242° (Meldola & Hollely, 1912); 3:5-diaminophenol, m.p. 180°, and its *NN'*-diacetyl derivative, m.p. 195° (Pollak, 1893); 2:6-diaminophenol sulphate, and its *ONN'*-tribenzoyl derivative, m.p. 220° (Fromm & Ebert, 1924); 2-amino-4-nitrophenol, m.p. 142°, and its *O*-toluene-*p*-sulphonate, m.p. 122° (Misslin & Bau, 1919); 4-amino-2-nitrophenol, m.p. 131°, and 4-acetamido-2-nitrophenol, m.p. 158° (Friedländer & Zeitlin, 1894); 3-amino-5-nitrophenol, m.p. 169°, and 3-acetamido-5-nitrophenol, m.p. 270° (decomp.) (Heller & Kammann, 1909); 2-amino-6-nitrophenol, m.p. 112°, and 2-acetamido-6-nitrophenol, m.p. 122° (Newberry & Phillips, 1928); *m*-nitrophenylhydroxylamine, m.p. 119° (Brand, 1905); *m*-nitrosonitrobenzene, m.p. 90° (Brand, 1905) (light-absorption in ethanol, max. 750 mμ, log ϵ, 1·8) (Nakamoto, 1952), and 4-bromo-3′-nitroazobenzene, m.p. 121° (Leandri & Risaliti, 1954); 3:3′-dinitroazoxybenzene, m.p. 146° (Lobry de Bruyn, 1893).

Animals. The *m*-dinitro[^{14}C]benzene contained about 25 μc/g. and was administered to rabbits by stomach tube as an aqueous suspension or by subdermal injection as a 10% (w/v) solution in arachis oil. The doses given were from 50 to 100 mg./kg. body wt. At the higher dose level (100 mg./kg.) the animals died on the third day. The rabbits were maintained on a daily diet of 100 g. of Diet 41 B (E. Dixon and Sons, Ware, Herts) and 100 ml. of water.

Measurement of radioactivity. Measurements were usually carried out on solid and liquid samples of 'infinite thickness' with an end-window counter tube. The specific activities were determined by comparison with a stable polymer reference (The Radiochemical Centre, Amersham, Bucks.) which was standardized against the *m*-dinitro[^{14}C]-benzene. The radioactivity of the urines and tissues was determined as described by Parke (1956).

Analytical methods. Glucuronic acid and ethereal sulphate in urine were determined as described by Mead, Smith & Williams (1958).

Paper chromatography. The possible metabolites of *m*-dinitrobenzene were mostly separable by chromatography in solvents *A* and *B* (see Table 1). The only exception was the separation of *m*-dinitrobenzene and *m*-nitrosonitrobenzene, which was not achieved with these or any other solvent system. However, these two substances could be distinguished by the colour reaction with *p*-dimethylaminocinnamaldehyde (see Table 1). *m*-Nitrophenylhydroxylamine was unstable and appeared to be atmospherically oxidized to *m*-nitrosonitrobenzene or 3:3′-dinitroazoxybenzene during chromatography.

Estimation of m-*nitroaniline and* m-*phenylenediamine.* *m*-Nitroaniline was estimated volumetrically with $TiCl_3$ by the method described by Robinson, Smith & Williams (1951), and *m*-phenylenediamine was estimated colorimetrically after diazotization. To 5 ml. of a solution con-

Table 1. *R_F values and colour reactions on paper of possible metabolites of* m-*dinitrobenzene*

R_F values are for descending chromatography on Whatman no. 1 paper, *A*, in benzene–acetic acid–water (1:1:2, by vol.) run for 6 hr., *B* in di*iso*propyl ether saturated with water (see Parke, 1960). Detecting reagents were: 0·1N-NaOH; 1% aq. $FeCl_3$; Gibbs's reagent consisting of 0·2% ethanolic soln. of 2:6-dichloroquinonechloroimide followed by 2N-Na_2CO_3; 0·02% soln. of *p*-dimethylaminocinnamaldehyde in ethanol containing 10% (v/v) of 2N-H_2SO_4; and reducing reagent consisting of the *p*-dimethylaminocinnamaldehyde soln. followed by a 1·5% (w/v) soln. of $Ti_2(SO_4)_3$ in N-H_2SO_4, which gave a violet colour with all the compounds listed below.

Compound	R_F values		Colour reactions			
	A	*B*	NaOH	$FeCl_3$	Gibbs's reagent	Dimethylamino-cinnamaldehyde
m-Dinitrobenzene	0·95	0·93	—	—	—	None
m-Nitrosonitrobenzene	0·96	0·93	—	—	—	Pale violet
m-Nitrophenylhydroxylamine	0·97	0·92	—	—	—	Pale violet
3:3′-Dinitroazoxybenzene	0·97	0·92	—	—	—	None
m-Nitroaniline	0·89	0·91	Orange	—	—	Violet
m-Phenylenediamine	0·05	0·20	—	—	—	Violet
2:4-Dinitrophenol	0·93	0·73	Yellow	None	Yellow	None
2:6-Dinitrophenol	0·91	0·71	Yellow	None	Yellow	None
3:5-Dinitrophenol	0·58	0·85	Yellow	None	Yellow	None
2-Amino-4-nitrophenol	0·08	0·75	Orange	Brown	Orange	Violet
2-Amino-6-nitrophenol	0·86	0·83	Orange	Brown	Violet	Violet
3-Amino-5-nitrophenol	0·07	0·74	Yellow	Buff	Violet	Violet
4-Amino-2-nitrophenol	0·56	0·82	Red	Brown	Violet	Violet
2:4-Diaminophenol	0·00	0·01	Blue	Red	—	Violet
2:6-Diaminophenol	0·00	0·03	Brown	Brown	—	Violet
3:5-Diaminophenol	0·00	0·00	Brown	Brown	—	Violet

taining 0·1–0·8 mg. of *m*-phenylenediamine in 0·5-HCl was added 1 ml. of a 0·1% (w/v) aq. solution of $NaNO_2$ followed by 2 ml. of 2N-Na_2CO_3; the mixture was diluted to 10 ml. and the light-absorption measured at 362 mμ. The standard curve is shown in Fig. 1. Recoveries of *m*-nitroaniline and *m*-phenylenediamine separately from aqueous solution were 95±5% and 96±5% respectively.

The total *m*-nitroaniline and *m*-phenylenediamine in solution in urines were estimated simultaneously by refluxing 25 ml. of urine with 12·5 ml. of conc. HCl for 3 hr. to hydrolyse the conjugates. The hydrolysed solutions were cooled, adjusted to pH 12·0 with 10N-NaOH and then extracted with three 20 ml. portions of ethyl acetate. The ethyl acetate extracts were bulked and extracted with three 10 ml. portions of 2N-HCl, and the acid extracts were bulked and diluted to 50 ml.; 5·0 ml. of this solution was used for the colorimetric determination of *m*-phenylenediamine, and 40 ml. was boiled with 5·0 ml. of $TiCl_3$ solution for the volumetric estimation of *m*-nitroaniline. Blank estimations were made on normal urines for 3 days before dosing with *m*-dinitrobenzene. Recoveries of mixtures of *m*-nitroaniline and *m*-phenylenediamine (25 mg./100 ml. each) from solutions in normal rabbit urine were 93±5% and 83±10% respectively. *m*-Nitroaniline also produces a colour on diazotization and the light-absorption at 362 mμ is shown in Fig. 1. Corrections were therefore made for the amount of *m*-nitroaniline present. The aminonitrophenols and 2:4-diaminophenol also produce a colour on diazotization and the aminonitrophenols consume $TiCl_3$, but since they are not extracted from aqueous solution at pH 12·0 they do not interfere in the estimations.

Estimation of metabolites

m-*Dinitrobenzene*. *m*-Dinitrobenzene (250 mg.) dissolved in 5 ml. of ethanol was added to a portion of the urine (10–20 ml.), and the solution was extracted with three portions of ether (10 ml.). The combined ethereal extracts were washed with N-HCl, N-NaOH, then water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue of *m*-dinitrobenzene was recrystallized from ethanol and toluene to constant specific activity (m.p. and mixed m.p. 91°).

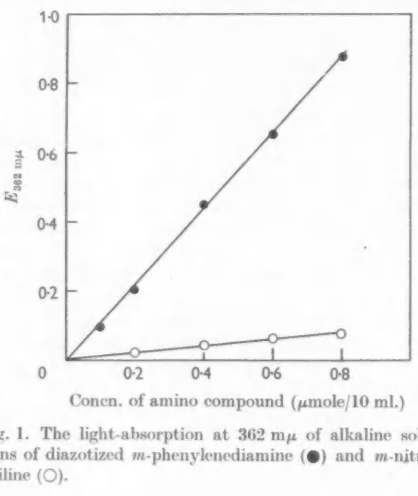

Fig. 1. The light-absorption at 362 mμ of alkaline solutions of diazotized *m*-phenylenediamine (●) and *m*-nitroaniline (○).

m-*Nitrosonitrobenzene*. *m*-Nitrosonitrobenzene (250 mg.) was added to a portion of the freshly voided urine (10 ml.) and the mixture steam-distilled. The nitrosonitrobenzene in the distillate was filtered off, washed with N-HCl to remove traces of *m*-nitroaniline, and recrystallized from ethanol and aqueous ethanol to constant specific radioactivity (m.p. and mixed m.p. 91°). The material was then converted into the 4-bromoazo compound (Ingold, 1925) by warming with an equimolecular amount of *p*-bromoaniline dissolved in 2 ml. of acetic acid. The mixture was poured into water and the orange 4-bromo-3′-nitroazobenzene filtered off and recrystallized from dilute acetic acid to constant specific radioactivity (m.p. and mixed m.p. 121°).

m-*Nitrophenylhydroxylamine*. *m*-Nitrophenylhydroxylamine (500 mg.) in 5 ml. of ethanol was added to a portion of the freshly voided urine and the mixture extracted with three portions of ether. The ethereal extracts were bulked, the ether was evaporated, and the residual *m*-nitrophenylhydroxylamine was recrystallized from benzene to constant specific radioactivity (m.p. and mixed m.p. 118°). The phenylhydroxylamine was then treated with 50 ml. of 10% aq. $FeCl_3$ soln., and steam-distilled to convert it into *m*-nitrosonitrobenzene, which was removed from the distillate by filtration, recrystallized from ethanol and aqueous ethanol (m.p. and mixed m.p. 91°) and its radioactivity determined.

3:3′-*Dinitroazoxybenzene*. 3:3′-Dinitroazoxybenzene (250 mg.) in 10 ml. of benzene was added to a portion of the freshly voided urine and the mixture shaken for 0·5 hr. to equilibrate. The mixture was then extracted with three portions of benzene, the benzene extracts were bulked, washed with N-NaOH, N-HCl and water, dried over anhydrous Na_2SO_4, and the benzene was removed. The residue was recrystallized from a mixture of ethanol and ethyl acetate, and then from formic acid to constant specific activity (m.p. and mixed m.p. 146°).

m-*Nitroaniline*. *m*-Nitroaniline (500 mg.) was added to a portion of the urine, which was then refluxed with an equal vol. of conc. HCl for 3 hr. The hydrolysed solution was adjusted to pH 8·0 with Na_2CO_3 and extracted with three portions of ether. The combined ethereal extracts were washed with water and evaporated to dryness, and the residue was steam-distilled. The *m*-nitroaniline in the steam-distillate was filtered off and recrystallized from water (m.p. and mixed m.p. 114°). It was finally dissolved in acetone (5 ml.) and treated with 1 equiv. of benzoyl chloride and excess of 2N-NaOH to give 3-nitrobenzanilide (m.p. and mixed m.p. 159°); this was recrystallized from pentanol to constant specific activity.

m-*Phenylenediamine*. *m*-Phenylenediamine (500 mg.) was added to a portion of the urine, which was then refluxed with an equal vol. of conc. HCl for 3 hr. The hydrolysed solution was adjusted to pH 8·0 with Na_2CO_3 and extracted with three portions of ethyl acetate. The combined extracts were washed with a little 0·2N-NaOH, and the *m*-phenylenediamine was transferred into 2N-HCl. The acid solution was then adjusted to pH 8·0 with Na_2CO_3 and extracted with three portions of ethyl acetate. The combined extracts were washed with a little 0·2N-NaOH, dried over anhydrous Na_2SO_4 and evaporated to dryness. The

residue was dissolved in water, filtered and treated with 2 equiv. of benzoyl chloride and excess of 2N-NaOH to yield the *NN'*-dibenzoyl derivative, which was recrystallized from acetic acid, methyl ethyl ketone and *cyclo*hexanone to constant specific activity (m.p. and mixed m.p. 240°).

Dinitrophenols. 2:4-, 2:6- and 3:5-Dinitrophenol (500 mg.) were separately added to portions of the urine, which were then refluxed with an equal vol. of conc. HCl for 3 hr. The solution of 2:4-dinitrophenol was cooled, the 2:4-dinitrophenol filtered off and dissolved in ether (20 ml.); the ethereal extract was washed with 2N-HCl, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was steam-distilled and the 2:4-dinitrophenol removed from the distillate by filtration and recrystallized from ethanol (m.p. 113°). The 2:4-dinitrophenol was then dissolved in acetone and treated with 1 equiv. of toluene-*p*-sulphonyl chloride and excess of 2N-NaOH to yield 2:4-dinitrophenyl toluene-*p*-sulphonate (m.p. and mixed m.p. 122°) which was recrystallized from ethanol to constant specific activity.

The solution containing 2:6-dinitrophenol was extracted with three portions of ether, and the combined ethereal extracts were washed with 2N-HCl, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was steam-distilled, the 2:6-dinitrophenol was extracted from the distillate with three portions of ether, and the ether evaporated. The residue of 2:6-dinitrophenol was recrystallized from ethanol, aq. ethanol and 0·1N-HCl until free from radioactivity (m.p. and mixed m.p. 61°).

The solution containing 3:5-dinitrophenol was extracted with three portions of ether and the combined ethereal extracts were washed with 2N-HCl. The dinitrophenol was then transferred into N-NaOH which was acidified with 2N-HCl and cooled. The precipitated dinitrophenol was filtered off, redissolved in N-NaOH, reprecipitated with 2N-HCl and collected by filtration. The 3:5-dinitrophenol was then recrystallized from 0·1N-HCl until free from radioactivity (m.p. and mixed m p. 126°).

Aminonitrophenols. 2-Amino-4-nitrophenol, 2-amino-6-nitrophenol, 3-amino-5-nitrophenol and 4-amino-2-nitrophenol (500 mg.) were separately added to portions of the urine which were then refluxed with an equal volume of conc. HCl for 3 hr. The hydrolysed solutions were made alkaline with 10N-NaOH and extracted once with ether, and the ether was discarded. The solutions were then adjusted to pH 7·0 with 2N-HCl and extracted with three portions of ether. The three ethereal extracts were bulked and washed once with water, and the aminonitrophenols were transferred into 2N-NaOH. Acidification of these solutions with 2N-HCl precipitates the aminonitrophenols, which are removed by filtration. The 2-amino-4-nitrophenol, m.p. 138°, was recrystallized from water and converted into its *O*-toluene-*p*-sulphonate, by treatment with one equivalent of toluene-*p*-sulphonyl chloride and excess of 2N-NaOH; it was then recrystallized from ethanol to constant activity (m.p. and mixed m.p. 122°). The three other aminonitrophenols were recrystallized from water, then dissolved in 2N-NaOH and treated with 1 equiv. of acetic anhydride. The solutions were acidified and the products filtered off and recrystallized from ethanol to constant specific activity (2-acetamido-6-nitrophenol, m.p. and mixed m.p. 122°; 3-acetamido-5-nitrophenol, m.p. 270° (decomp.); and 4-acetamido-2-nitrophenol, m.p. 157°).

In some experiments 4-amino-2-nitrophenol was determined after hydrolysis of the urine with a crude preparation of β-glucuronidase and arylsulphatase. The urine (10 ml.) was adjusted to pH 4·6 with acetic acid and then incubated at 30° for 16 hr. with 1 ml. of crop fluid from *Helix pomatia* (see Leon, Bulbrook & Corner, 1960). Carrier 4-amino-2-nitrophenol (5–10 mg.) was then added to the mixture, which was again adjusted to pH 4·6, and incubated for a further 24 hr. after addition of another 1 ml. of the glucuronidase preparation. The mixture was then adjusted to pH 8·0 and extracted twice with 10 ml. portions of ether. The ethereal extracts were concentrated and chromatographed on Whatman no. 3MM paper in solvent mixture *B*. The band corresponding to 4-amino-2-nitrophenol was cut out and eluted with acetone, and the eluate rechromatographed on Whatman no. 1 paper in solvent *A*. The 4-amino-2-nitrophenol band was again cut out and eluted with acetone, and the eluate evaporated on a planchet; the residue was weighed and the radioactivity determined with an infinitely thin sample. The specific radioactivity remained constant after further chromatography in solvent *A*.

Diaminophenols. 3:5-Diaminophenol (500 mg.), 2:4-diaminophenol dihydrochloride (1 g.) and 2:6-diaminophenol sulphate (1 g.) were separately added to portions of the urine which were then refluxed for 3 hr. with an equal vol. of conc. HCl. The hydrolysed solutions were adjusted to pH 8·5 with Na_2CO_3 and extracted with three portions of butan-1-ol. The butanol extracts were bulked, and the diaminophenols were transferred into 2N-HCl. The acid solutions were adjusted to pH 8·5 with Na_2CO_3 and again extracted with butan-1-ol, and the diaminophenols transferred to 2N-HCl. The acid solutions were washed with ether and the ethereal extracts discarded. The solutions of 2:4- and 2:6-diaminophenol were then treated with excess of benzoyl chloride, solid Na_2SO_3 and 2N-NaOH to give the *ONN'*-tribenzoyl derivatives; the solution of 3:5-diaminophenol was treated with excess of acetic anhydride and 2N-NaOH to give the *NN'*-diacetamidophenol. 2:4-Diaminophenol tribenzoate was recrystallized from ethyl acetate to constant specific activity (m.p. and mixed m.p. 241°). 2:6-Diaminophenol tribenzoate (m.p. and mixed m.p. 220°) was recrystallized from ethanol and then ethyl acetate and 3:5-diacetamidophenol (m.p. and mixed m.p. 195°) was recrystallized from ethanol, until free from radioactivity.

Chromatographic detection of metabolites

The urine from a rabbit dosed with *m*-dinitro[^{14}C]benzene (Expt. 3), which remained after isotope dilution estimations, was hydrolysed by refluxing with an equal volume of conc. HCl for 3 hr. The hydrolysed urine was then continuously extracted with ether and twice again extracted with ether after adjusting to pH 7·0 and then 9·0 with Na_2CO_3. The three extracts were chromatographed on Whatman no. 1 paper in solvents *A* and *B*, and radioautographs were subsequently obtained.

The extract from acid solution showed radioactive bands with colour reactions and R_F values corresponding to *m*-dinitrobenzene and 3:3'-dinitroazoxybenzene, each present to about 1% of the dose, and 2:4-dinitrophenol (0·1% of dose). In addition a dark-brown band (15–20% of the dose) was present at the origin of the chromatograms in both solvents, and probably consisted of polymeric

oxidation products of *m*-phenylenediamine and diaminophenol. 2:6- and 3:5-Dinitrophenol were not present in amounts > 0·02% of the dose. The extract at pH 7·0 showed radioactive bands with colour reactions and R_F values corresponding to *m*-phenylenediamine (10% of dose), 2-amino-4-nitrophenol (10% of dose), *m*-nitroaniline (5% of dose), 4-amino-2-nitrophenol (1% of dose), and a band at the origin in both solvents (10% of dose) consisting of oxidation products of *m*-phenylenediamine and diaminophenol. 2-Amino-6-nitrophenol and 3-amino-5-nitrophenol were not present in amounts > 0·05% of the dose. The extract at pH 9·0 showed only two radioactive bands, one at the origin (25% of dose) and one corresponding to *m*-nitroaniline (3% of dose).

Isolation of metabolites from unhydrolysed urine

m-*Nitroaniline*. *m*-Dinitrobenzene (0·25 g.) in arachis oil (5 ml.) was administered orally to each of two rabbits. The urines, which were collected for 2 days, were dark brown, had pH 7·4, did not reduce Benedict's reagent but gave a strong positive naphtharesorcinol reaction for glucuronides. They frequently contained albumin, erythrocytes, epithelial cells and cellular casts. The combined urines were adjusted to pH 2 with 2N-HCl and then continuously extracted with ether for 6 hr.; the ethereal extract was concentrated to 20 ml. and extracted three times with 10 ml. portions of 2N-HCl. The acid extracts were bulked, washed with 20 ml. of ether, adjusted to pH 10 with 10N-NaOH and then extracted three times with 10 ml. portions of ether. The bulked ethereal extracts were washed with water (10 ml.) and then extracted twice with 5 ml. portions of 5N-HCl. The acid extracts were adjusted to pH 10 with 10N-NaOH, and, on cooling, crystals of *m*-nitroaniline were deposited (yield, 43 mg. equivalent to 9% of dose) which after recrystallization from aqueous ethanol had m.p. 113°, mixed m.p. 114°. Benzoylation of this material with benzoyl chloride and 2N-NaOH gave 3-nitrobenzanilide, m.p. and mixed m.p. 159°.

3:3′-*Dinitroazoxybenzene and* m-*dinitrobenzene*. The ethereal extracts of the urines remaining after extraction with 2N-HCl and the ether washings of the acid extracts were bulked, washed with 2N-NaOH and water and evaporated to dryness. The residue (125 mg.) was digested with benzene, and the benzene solution was filtered and evaporated to dryness. The residue (113 mg.) gave a deep-violet colour with acetone and NaOH, indicative of *m*-dinitrobenzene or *m*-nitrosonitrobenzene, and was dissolved in 5 ml. of hot ethanol. On cooling, the solution deposited a few dark-brown crystals, m.p. 138° (1·5 mg., equivalent to 0·3% of dose) which on recrystallization from formic acid gave 3:3′-dinitroazoxybenzene (m.p. 142°, mixed m.p. 144°). The ethanolic solution was diluted with water and the precipitate (12 mg., 2·5% of the dose) which was deposited was filtered off and recrystallized repeatedly from aqueous ethanol to yield colourless crystals of *m*-dinitrobenzene, m.p. 90° (admixture of *m*-nitrosonitrobenzene with *m*-dinitrobenzene in the proportions of 0·5, 1 and 2 to 1 does not significantly depress the m.p. from 89–90°). The eutectic of *m*-nitrosonitrobenzene (25%) and *m*-dinitrobenzene (75%) is distinctly green (Nakamoto, 1952).

m-*Nitrosonitrobenzene*. The urines from six rabbits fed a total of 1·5 g. of *m*-dinitrobenzene were collected for 2 days, bulked, adjusted to pH 6·0 and steam-distilled. The steam-distillate was extracted with ether, and the extract dried over anhydrous Na_2SO_4 and evaporated to give a non-crystalline residue, which by paper chromatography was shown to contain *m*-nitroaniline and a trace of *m*-dinitrobenzene. The urine was again steam-distilled after addition of 20 g. of $FeCl_3,6H_2O$, and the steam-distillate extracted with ether; the extract was dried over anhydrous Na_2SO_4 and evaporated. The residual trace of material was recrystallized from a little aqueous ethanol to give a few crystals (< 1 mg.) (m.p. 89°, mixed m.p. with *m*-nitrosonitrobenzene 90°) which were green in liquid state and on paper gave a violet colour with *p*-dimethylaminocinnamaldehyde alone. This material by virtue of its m.p., colour and colour reaction was most probably *m*-nitrosonitrobenzene, formed from *m*-nitrophenylhydroxylamine by the oxidation with $FeCl_3$.

Isolation of metabolites from hydrolysed urine

2:4-*Dinitrophenol*. The combined urines from two rabbits which had been fed a total of 0·5 g. of *m*-dinitrobenzene were hydrolysed by refluxing for 3 hr. with an equal volume of conc. HCl. The urines were then cooled and continuously extracted with ether, and the ethereal extract was washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue (220 mg.) on chromatography on paper in solvents *A* and *B* revealed the presence of trace amounts (equivalent to about 0·1% of dose) of 2:4-dinitrophenol and *m*-nitroaniline. 2:6- and 3:5-Dinitrophenol were not present in amounts greater than 0·02% of the dose. The residue was redissolved in ether, washed with 2N-HCl, and the ether solution dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was digested with hot water and on cooling the solution deposited a few crystals (1 mg.), m.p. 114°, not depressed on admixture with 2:4-dinitrophenol. Paper chromatography of an ethereal extract of the HCl washings revealed only *m*-nitroaniline.

The absence of significant amounts of dinitrophenols was confirmed by u.v.-absorption spectra. The urines obtained from two rabbits dosed orally with a total of 0·5 g. of *m*-dinitrobenzene, collected over 2 days, were bulked and hydrolysed by refluxing for 3 hr. with 0·5 vol. of conc. HCl. The hydrolysed urine was cooled and continuously extracted with ether for 8 hr. The ethereal extract (50 ml.) was extracted with two 20 ml. portions of 2N-NaOH, and the alkaline extracts were bulked, washed with ether, acidified with conc. HCl and extracted with two 20 ml. portions of ether. The ethereal extract was washed with water, dried over anhydrous Na_2SO_4, and evaporated to dryness at room temperature. The residue (about 10 mg.) was dissolved in 10 ml. of 0·1N-NaOH, filtered and diluted 1 to 10 with 0·1N-NaOH and 0·1N-HCl. The absorption spectra of these solutions were determined with a Unicam SP. 500 spectrophotometer and compared with the absorption spectra of 2:4-, 2:6- and 3:5-dinitrophenol in 0·1N-HCl and 0·1N-NaOH (see Figs. 2, 3). From the absorption spectra it is seen that no significant amounts of dinitrophenols were present, and 2:4-dinitrophenol was estimated to be present in an amount not greater than 0·1% of the dose. Flexser, Hammett & Dingwall (1935) showed that 2:4-dinitrophenol has light-absorption max. 261 mμ (ϵ 11 200) in 0·1N-HCl; and 364 mμ (ϵ 14 800) in 0·1N-NaOH.

2-*Amino*-4-*nitrophenol*. The hydrolysed urine after ether extraction of 2:4-dinitrophenol was then adjusted to

pH 8·0 with solid Na_2CO_3 and continuously extracted with ether. The ethereal extract (50 ml.) was extracted twice with 20 ml. portions of 2N-HCl; the acid extracts were combined, washed with ether, adjusted to pH 8·0 with Na_2CO_3 and extracted with three 20 ml. portions of ether. The ethereal extracts were bulked, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue (215 mg.) was digested with hot water and filtered, and, on cooling the solution, red crystals, m.p. 70° (28 mg., equivalent to 5% of the dose) were deposited. Chromatography of this material on paper in solvents *A* and *B* revealed three spots with R_F values and colour reactions corresponding to 2-amino-4-nitrophenol, 4-amino-2-nitrophenol, and *m*-nitroaniline. The bulk of the crystalline material was chromatographed on Whatman no. 3 MM paper in solvent *B* and a broad orange band containing the two aminonitrophenols (R_F 0·7–0·85) and a yellow band (R_F 0·9–0·95) containing *m*-nitroaniline were cut out and eluted with ether. The material from the first band was rechromatographed on Whatman no. 1 paper in solvent *A* and gave a major band with R_F 0·08 and a minor one with R_F 0·56. The major band was cut out and eluted with ether; the ether was evaporated and the residue recrystallized from water to give 2-amino-4-nitrophenol, m.p. and mixed m.p. 143° (16 mg., equivalent to 3% of the dose). Treatment with 1 equiv. of toluene-*p*-sulphonyl chloride and excess of 2N-NaOH gave the *O*-toluene-*p*-sulphonate, m.p. and mixed m.p. 122°. Elution of the minor band gave a trace of red material which did not crystallize and did not yield a crystalline derivative on treatment with acetic anhydride and excess of 2N-NaOH; light-absorption max., 239 and 495 mμ in N-NaOH and 267 and 340 mμ in N-HCl, indicative of 4-amino-2-nitrophenol [light-absorption max. 239, ~295 and 500 mμ (ε 16 300, 4100 and 3800 respectively) in N-NaOH and 267 and 340–345 mμ (ε 5400 and 2800) in N-HCl, cf. Parker (1952)].

m-Phenylenediamine. The combined urines collected over 2 days from two rabbits fed a total of 0·5 g. of *m*-dinitrobenzene were hydrolysed by refluxing with an equal volume of conc. HCl for 3 hr. The hydrolysed urines were adjusted to pH 8·0 with solid Na_2CO_3 and extracted three times with 0·5 vol. of ethyl acetate. The extracts were combined and in turn extracted twice with 25 ml. of 2N-HCl. The acid extracts were adjusted to pH 8·0 with solid Na_2CO_3 and extracted twice with 25 ml. of ethyl acetate. The ethyl acetate extracts were dried over anhydrous Na_2SO_4, concentrated *in vacuo*, and the residue was chromatographed on Whatman no. 3 MM paper in solvent *B*. The band with R_F 0·20, corresponding to *m*-phenylenediamine, was cut out, eluted with N-HCl, and the eluate treated with an excess of benzoyl chloride and 2N-NaOH to yield *NN'*-dibenzoyl-*m*-phenylenediamine (yield 70 mg., equivalent to 6% of the dose), m.p. and mixed m.p. 240° after recrystallization from ethanol and acetic acid.

2:4-*Diaminophenol.* The combined urines collected over 24 hr. from two rabbits fed a total of 0·5 g. *m*-dinitrobenzene were heated on a boiling-water bath for 0·5 hr. after addition of 0·1 vol. of conc. HCl. The partially hydrolysed urine was cooled and continuously extracted with ether for 8-hr. periods. The ethereal extract of the first 8 hr. period was evaporated to dryness, and the residue was

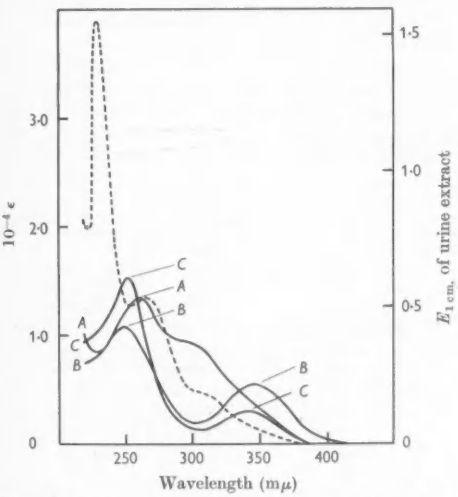

Fig. 2. The absorption spectrum in 0·1 N-HCl (100 ml.) of an extract of urine from rabbits after an oral dose (500 mg.) of *m*-dinitrobenzene (- - -), compared with the spectra of 2:4-, 2:6- and 3:5-dinitrophenol in 0·1N-HCl. *A*, 2:4-Dinitrophenol ($\lambda_{max.}$ 261, ~300 mμ; $\epsilon_{max.}$ 13 600, 9400); *B*, 2:6-dinitrophenol ($\lambda_{max.}$ 248, 345 mμ; $\epsilon_{max.}$ 10 800, 5400); and *C*, 3:5-dinitrophenol ($\lambda_{max.}$ 252, 340 mμ; $\epsilon_{max.}$ 15 300, 3100).

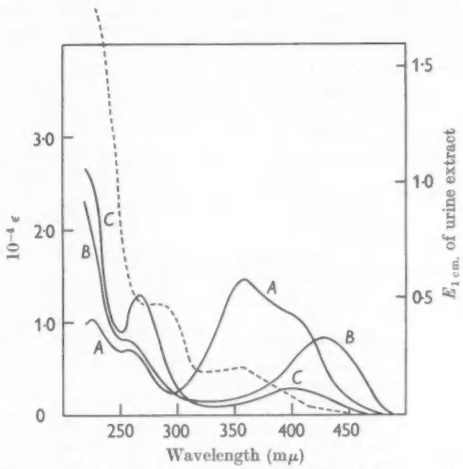

Fig. 3. The absorption spectrum in 0·1N-NaOH (100 ml.) of an extract of urine from rabbits after an oral dose (500 mg.) of *m*-dinitrobenzene (- - -), compared with the spectra of 2:4-, 2:6- and 3:5-dinitrophenol in 0·1N-NaOH. *A*, 2:4-Dinitrophenol ($\lambda_{max.}$ 226, 258, 358 mμ; $\epsilon_{max.}$ 10 300, 7200, 14 800); *B*, 2:6-dinitrophenol ($\lambda_{max.}$ ~258, 428 mμ; $\epsilon_{max.}$ 8000, 8200); *C*, 3:5-dinitrophenol ($\lambda_{max.}$ 268, 400 mμ; $\epsilon_{max.}$ 13 000, 2800).

digested with water (20 ml.) and acidified by addition of a few drops of 2N-acetic acid to which a crystal or two of Na_2SO_3 was added. The aqueous solution was filtered and treated with an excess of a saturated solution of lead acetate. The ppt. which formed (mostly the lead salt of 2-amino-4-nitrophenol) was filtered off, and the filtrate was adjusted to pH 8·0 with aq. 2N-NH_3 soln. and treated with an excess of a saturated solution of basic lead acetate (2:4-diaminophenol forms an insoluble salt with basic lead acetate but not with lead acetate). The ppt. formed was deposited by centrifuging, washed with water, suspended in water, and the suspension saturated with H_2S. The precipitated PbS was filtered off and the filtrate evaporated to dryness *in vacuo*. The residue (about 20 mg.) was treated with 0·1 ml. of benzoyl chloride and an excess of solid Na_2SO_3, and finally made alkaline with solid Na_2CO_3, but no crystalline material was obtained. The ethereal extract of the second 8 hr. period was treated similarly. No ppt. was obtained with lead acetate solution, but a substantial ppt. was formed after addition of basic lead acetate. The residue finally obtained after removal of the lead and evaporation was dissolved in 5 ml. of water. The solution gave a red colour with Br_2 water or 10% (w/v) aq. $FeCl_3$ soln., and a blue colour with 2N-NaOH, which are colour reactions given by 2:4-diaminophenol. The solution was treated with 0·1 ml. of benzoyl chloride and an excess of solid Na_2SO_3 and finally made alkaline with 2N-NaOH, to yield a small amount of crystalline material (2 mg., equivalent to 0·1% of dose) which was washed with water and recrystallized from a mixture of ethanol and ethyl acetate to give the *ON N'*-tribenzoate of 2:4-diaminophenol (m.p. and mixed m.p. 240°). The very small yield of 2:4-diaminophenol isolated is probably due to its facile oxidation; Ogino & Yasukura (1957) who isolated 2:4-diaminophenol from the urine of rabbits dosed with 2:4-dinitrophenol were able to obtain only small amounts of material, equivalent to 0·2% of the dose. Attempts at the isolation of 2:4-diaminophenol from urine hydrolysed by refluxing for 3 hr. with an equal volume of conc. HCl yielded no crystalline material.

RESULTS AND DISCUSSION

After an oral dose of *m*-dinitro[^{14}C]benzene some 65–93% is excreted in the urine in 2 days (see Table 2), and subsequently only trace amounts of radioactivity are found in the urine. The faeces contain only small amounts of radioactivity (1–5% of dose), and the rest of the radioactivity is presumably retained in the tissues, from which it is slowly excreted in the urine. The radioactivity of the tissues could not be accurately determined, owing to the low specific activity of the administered *m*-dinitrobenzene. The mean values for urine conjugates for three rabbits dosed orally with *m*-dinitrobenzene (80 mg./kg. body wt.) were: glucuronides, 30% of dose (range 21–41%); and ethereal sulphates, 6% of dose (range 0–11%).

The major metabolites of *m*-dinitrobenzene found in the urine were *m*-nitroaniline and *m*-phenylenediamine, which together amount to 35% of the dose, 2:4-diaminophenol and 2-amino-4-nitrophenol. The minor metabolites present were unchanged *m*-dinitrobenzene, 2:4-dinitrophenol, 4-amino-2-nitrophenol, *m*-nitrophenylhydroxylamine and artifacts derived from this, 3:3′-dinitroazoxybenzene and *m*-nitrosonitrobenzene. 2:6- and 3:5-Dinitrophenol, 2:6- and 3:5-diaminophenol, 2-amino-6-nitrophenol and 3-amino-5-nitrophenol were not present in amounts greater than 0·05% of the dose. All the metabolites estimated by isotope dilution procedures, with the exception of 4-amino-2-nitrophenol and *m*-nitrophenylhydroxylamine, have been isolated. The presence of 4-amino-2-nitrophenol has been confirmed by paper chromatography and u.v.-absorption spectra; the presence of *m*-nitrophenylhydroxylamine, which is

Table 2. *Metabolites of* m-*dinitro*[^{14}C]*benzene excreted in urine by rabbits*

m-Dinitrobenzene was administered orally as aqueous suspensions. Urines were collected for 2 days.

Expt. no.	1*	2	3	4	5	6	7	
Dose of *m*-dinitrobenzene (mg./kg.)	100	70	70	60	60	50	50	
Dose of ^{14}C (μc/animal)	5	4	5	4	4	2	3	Average
	Percentage of dose							
m-Dinitrobenzene	<0·1	0·2	—	2·4	—	0·4	0·7	0·7
m-Nitrophenylhydroxylamine	—	—	—	—	0·7	1·1	0·75	0·8
m-Nitrosonitrobenzene	—	—	—	0·5	0·2	0·1	0·25	0·25
3:3′-Dinitroazoxybenzene	—	—	—	0·3	0·2	0·3	—	0·3
m-Nitroaniline (total)	28	35	10	18	—	—	—	} 35
m-Phenylenediamine (total)	<0·2	<0·2	23	25	—	—	—	
2:4-Dinitrophenol (total)	0·1	0·1	<0·1	—	—	—	—	0·1
2-Amino-4-nitrophenol (total)	12·5	15	12	16	—	—	—	14
4-Amino-2-nitrophenol (total)	1·4	1·6	2·1	2·6	—	1·9 (0·6)†	2·4 (1·0)†	2·0 (0·8)†
2:4-Diaminophenol (total)	19	37	38	28	—	—	—	31
Total metabolites	61	89	85	93	—	—	—	83
Total radioactivity in urine	65	89	82	93	71	89	75	81
Total radioactivity in faeces	—	0·3	1·0	—	5·2	—	—	—

* Animal died on 3rd day.

† Estimated after enzymic hydrolysis.

readily oxidized to 3:3′-dinitroazoxybenzene in alkaline solution and to *m*-nitrosonitrobenzene in neutral and acid media, has been indirectly confirmed by the isolation of both of these, the latter after oxidation with $FeCl_3$. *m*-Nitrophenylhydroxylamine and 3:3′-dinitroazoxybenzene were both estimated in freshly voided urine, but the possibility of their being artifacts arising from oxidation of *m*-nitrophenylhydroxylamine during the estimation procedures cannot be eliminated.

The excretion of traces of unchanged *m*-dinitrobenzene is surprising, for at similar doses neither benzene (Parke & Williams, 1950) nor nitrobenzene (Parke, 1956), both of which are more soluble than *m*-dinitrobenzene in water, is excreted unchanged in the urine. Possibly the excretion of traces of the unchanged material is due to the kidney damage which was indicated in many of the animals.

Reduction products. The excretion of some 1% of the dose as *m*-nitrophenylhydroxylamine or its artifacts confirms the findings of Lipschitz (1920) and Comel (1931) that *m*-nitrophenylhydroxylamine is formed from *m*-dinitrobenzene in animal tissues. A considerable amount of dinitrohydroxylaminotoluene was isolated from the urine of rabbits dosed with trinitrotoluene (Channon, Mills & Williams, 1944), but in the metabolism of nitrobenzene no trace of phenylhydroxylamine nor any artifact of it was found (Parke, 1956). Jackson & Waters (1958) have demonstrated in non-biological systems that free radicals may catalyse hydrogen transfer with *m*-dinitrobenzene or *s*-trinitrobenzene as oxidizing agents. In such reductions, Jackson & Waters (1960), using benzyl radicals, have shown that the free radicals react readily with trinitrobenzene to give a dinitrophenylhydroxylamine derivative, less readily with *m*-dinitrobenzene, to give small amounts of the *m*-nitrophenylhydroxylamine derivative together with *m*-nitroaniline, and with nitrobenzene to give only aniline. This is qualitatively and quantitatively similar to the metabolic reductions of the aromatic nitro compounds, and it is possible that the biological reduction of these compounds may be effected by a similar free radical mechanism involving flavins. The nitro-reductases described by Fouts & Brodie (1957), Bueding & Jolliffe (1946) and Müller (1958) are considered to be flavoproteins, which may be replaced by flavin mononucleotide, flavinadenine dinucleotide and even riboflavin; and free radical flavin semiquinones have been shown to arise as intermediates of the flavoprotein acyl dehydrogenases (Beinert, 1957; Beinert & Page, 1957; Ke, 1957).

The two other reduction products of *m*-dinitrobenzene found in the urine, *m*-nitroaniline and *m*-phenylenediamine, together account for about 35% of the dose. The former usually amounts to 14% and the latter to 21%, but in the first two experiments with *m*-dinitro[^{14}C]benzene no significant amount of *m*-phenylenediamine could be detected, and the amounts of *m*-nitroaniline were approximately equal to the total of the two reduction products of subsequent experiments. The reduction of only one of the nitro groups of *m*-dinitrobenzene has been observed in polarographic studies of the blood of dogs fed *m*-dinitrobenzene, reduction to *m*-nitroaniline occurring within the erythrocytes (Roubal, Tuný & Pokorný, 1946). In two different breeds of rabbit dosed with unlabelled *m*-dinitrobenzene (Table 3) both *m*-nitroaniline and *m*-phenylenediamine were formed in

Table 3. *Excretion of* m-*nitroaniline and* m-*phenylenediamine by rabbits after dosage with* m-*dinitrobenzene*

The dose of *m*-dinitrobenzene was 80 mg./kg. Urines were collected for 2 days. The mean values and standard errors of the mean are given in parentheses.

Breed of rabbit	Route of administration	Percentage of dose excreted as *m*-Nitroaniline	Percentage of dose excreted as *m*-Phenylenediamine
English	Oral	14	16
English	Oral	10 (14±3)	12 (17±3)
English	Subcutaneous	19	22
Chinchilla	Oral	9	18
Chinchilla	Oral	16 (14±2)	23 (21±2)
Chinchilla	Subcutaneous	16	23
Chinchilla	Oral*	9	12
Chinchilla	Oral*	15 (12±2)	16 (13±2)
Chinchilla	Subcutaneous*	11	10
Chinchilla	Oral*†	14	11
Chinchilla‡	Subcutaneous*†	14 (12±2)	9 (8±2)
Chinchilla‡	Subcutaneous*†	8	5

* Aureomycin (30 mg.) administered orally 12 hr. before dosage with *m*-dinitrobenzene and then again at 12 hr. intervals.

† *m*-Dinitrobenzene (80 mg./kg.) without aureomycin was administered orally 14 days previously.

‡ These animals died 20–24 hr. after dosing.

amounts similar to those found in Expts. 3 and 4 with *m*-dinitro[^{14}C]benzene. The subcutaneous administration of *m*-dinitrobenzene did not lessen the amounts of the reduction products appearing in the urine, from which it may be inferred that neither compound arises to any extent from reduction of the *m*-dinitrobenzene by intestinal bacteria (cf. Bray, Hybs, James & Thorpe, 1953). The simultaneous administration of aureomycin has no effect on the reduction of the first nitro group of *m*-dinitrobenzene, which is in agreement with the observations of Fouts & Brodie (1957) that reduction of *m*-dinitrobenzene and other aromatic nitro compounds by rabbit-liver nitro-reductase is not inhibited by aureomycin; reduction of *p*-nitrobenzaldehyde by rat liver is, however, inhibited by the antibiotic (Green, Josimovich, Tsou & Seligman, 1956). The extent of the reduction of *m*-dinitrobenzene to *m*-phenylenediamine seems to be inhibited, however, especially when the animals had been dosed with *m*-dinitrobenzene 2 weeks earlier, and the toxic effects were much more severe, two of the animals dying at the end of the first day. Fouts & Brodie (1957) have shown that in rabbit liver there are at least two systems reducing aromatic nitro groups, one reducing nitrophenols and another reducing non-phenolic compounds. This would explain a second anomaly of Expts. 1 and 2, for despite the absence of *m*-phenylenediamine, 2:4-diaminophenol was present to about the same extent as in Expts. 3 and 4, where both reduction products were formed. The anomalous absence of *m*-phenylenediamine in two experiments, and the effect of aureomycin on the reduction of the second nitro group only, suggests that the reduction of the two nitro groups of *m*-dinitrobenzene involves at least two different systems, enzymic or otherwise, whereas the reduction of dinitrophenols involves at least one more.

Phenols. The only dinitrophenol detected in the urine of rabbits dosed with *m*-dinitrobenzene was 2:4-dinitrophenol, which was present in only trace amounts (0·1% of dose). This is in marked contrast to nitrobenzene which is metabolized by the rabbit to give 18% of the dose as nitrophenols (Parke, 1956). Since 2:4-dinitrophenol administered to rats is excreted partly unchanged (Parker, 1952) it would seem that the direct hydroxylation of *m*-dinitrobenzene is only a minor process. Alternatively, the 2:4-dinitrophenol formed by hydroxylation of *m*-dinitrobenzene *in situ* may be almost completely metabolized to aminonitrophenols and diaminophenols, at sites not readily accessible to dinitrophenol administered by injection. The aminonitrophenol metabolites of *m*-dinitrobenzene are 2-amino-4-nitrophenol (14% of dose) and 4-amino-2-nitrophenol (0·8% of dose). Parker (1952) found that although 2:4-dinitrophenol was reduced by rat-liver homogenates to 2-amino-4-nitrophenol (10%) and 4-amino-2-nitrophenol (90%), the latter is further metabolized more rapidly, probably to 2:4-diaminophenol, so that only the 2-amino-4-nitrophenol is excreted in the urine of rats injected with 2:4-dinitrophenol. However, both aminonitrophenols were found in the urine and tissues of dogs injected with 2:4-dinitrophenol (Guerbet & Mayer, 1932). The 4-amino-2-nitrophenol detected and estimated after acid hydrolysis could have been an artifact of *m*-nitrophenylhydroxylamine, which is converted into the aminonitrophenol on treatment with strong mineral acids. Estimations of 4-amino-2-nitrophenol after hydrolysis of conjugates with snail crop-fluid as a source of arylsulphatase and β-glucuronidase show that it is a true metabolite, and the difference between the estimations after enzymic hydrolysis (0·8% of dose) and acid hydrolysis (2·0% of dose) is approximately equal to the amount of *m*-nitrophenylhydroxylamine present (0·9%). This is additional evidence of the presence of *m*-nitrophenylhydroxylamine. 2:4-Diaminophenol is the only diaminophenol present and is the major phenolic metabolite of *m*-dinitrobenzene. It has been isolated from the urine of rabbits injected with 2:4-dinitrophenol and is itself converted in the animal into 2-amino-4-quinoneimine, which is bound to tissue protein and causes cataract (Ogino & Yasukura, 1957). Some of the radioactivity remaining in the tissues is probably present in this form.

SUMMARY

1. The metabolism of *m*-dinitro[^{14}C]benzene has been studied in the rabbit at oral doses of 50–100 mg./kg.; 65–93% of the dose is excreted in the urine during the first two days and 1–5% in the faeces.

2. The principal metabolites in the urine are 2:4-diaminophenol (31% of dose), *m*-phenylenediamine (21%), *m*-nitroaniline (14%), and 2-amino-4-nitrophenol (14%). Traces of unchanged *m*-dinitrobenzene (0·7%), 2:4-dinitrophenol (0·1%), 4-amino-2-nitrophenol (0·8%), *m*-nitrophenylhydroxylamine (0·8%), *m*-nitrosonitrobenzene (0·25%) and 3:3′-dinitroazoxybenzene (0·3%) are also present. 2:6- and 3:5-Dinitrophenol, 2-amino 6-nitrophenol and 3-amino-5-nitrophenol, and 2:6- and 3:5-diaminophenol, are not present in amounts greater than 0·05% of the dose.

3. With oral doses of non-radioactive *m*-dinitrobenzene, an average of 30% of the dose is excreted as glucuronide conjugates and 6% as ethereal sulphates.

4. 2:4-Diaminophenol, *m*-phenylenediamine, *m*-nitroaniline, 2-amino-4-nitrophenol, 2:4-dinitrophenol, 3:3′-dinitroazoxybenzene, *m*-nitrosonitro-

benzene, and unchanged *m*-dinitrobenzene, or derivatives of these, have been isolated from the urine of rabbits dosed with *m*-dinitrobenzene. The presence of 4-amino-2-nitrophenol has been confirmed by paper chromatography and ultraviolet-absorption spectra.

5. The ultraviolet-absorption spectra of 2:4- and 2:6- and 3:5-dinitrophenol in 0·1 N-hydrochloric acid and 0·1 N-sodium hydroxide are recorded, and the absence of these dinitrophenols except for traces of 2:4-dinitrophenol (0·1% of dose) is confirmed from ultraviolet spectra of extracts of the hydrolysed urine from rabbits dosed with *m*-dinitrobenzene.

6. 4-Amino-2-nitrophenol was estimated after acid hydrolysis (2·0% of dose), and after enzymic hydrolysis (0·8% of dose). The estimation after acid hydrolysis includes 4-amino-2-nitrophenol arising as an artifact of 3-nitrophenylhydroxylamine (0·8% of dose).

7. In two experiments with *m*-dinitro[^{14}C]-benzene, no *m*-phenylenediamine was formed and the amounts of *m*-nitroaniline present in the urine were approximately equal to the sum of the two reduction products (35% of dose) formed in other experiments.

8. The reduction of *m*-dinitrobenzene to *m*-phenylenediamine is inhibited by aureomycin.

9. A mechanism for the reduction of aromatic nitro compounds involving flavin free radicals is postulated.

I am indebted to Professor R. T. Williams for his constant interest in this work.

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Metabolically Inert Proteins of the Central and Peripheral Nervous System, Muscle and Tendon

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It has recently been suggested that brain constituents, in common with the constituents of other tissues, may be roughly divided into at least two main groups according to their predominant rates of turnover (Davison & Dobbing, 1959). These two groups are represented by a metabolically active compartment and a more permanent compartment associated with structural elements such as the myelin sheath. Evidence in support of this concept comes from studies of lipid metabolism *in vivo* in the central nervous system. It was shown by labelling with ^{14}C and ^{32}P that part of the cerebral cholesterol and phosphatide undergoes turnover whereas the remainder is metabolically stable (Davison, Morgan, Wajda & Payling Wright, 1959; Davison & Dobbing, 1959, 1960*a*, *b*). Lipid undergoing turnover was found mainly in the grey matter, whereas the metabolically inert lipid was located predominantly in the white matter (Davison & Dobbing, 1960*b*; Davison, Dobbing, Morgan & Payling Wright, 1959). Furthermore, radioactivity was found to persist in those lipids which are characteristic of the myelin sheath.

It is known that the myelin sheath contained protein and lipid arranged as laminae around the axon (Finean & Robertson, 1958), and since the lipids of the myelin sheath appear to be inert it seemed possible that these myelin proteins may also be metabolically stable. Since at least a part of the myelin protein is of proteolipid nature (Folch & Lees, 1951; Koenig, 1959), it seemed of interest to investigate the metabolism of neural proteolipids and other proteins by using [1-^{14}C]-glycine as a suitable precursor for proteolipid protein (Folch & Lees, 1951; Uzman, 1958; Uzman & Rosen, 1958) and for other constituents.

In the present study radioactive glycine was injected into developing rats and became incorporated into both non-structural and structural elements, such as the myelin sheath and tendon. The fate of the labelled proteins was followed at intervals up to 250 days after the initial injection, by which time the rats were fully grown. By this means it was possible to distinguish between metabolically active and stable groups of protein.

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Adult rats were also given a single injection of [1-^{14}C]glycine, and the turnover of labelled protein was followed for 197 days. In conformity with the present hypothesis it was found that in the adult all the labelled proteins were metabolically active.

MATERIALS AND METHODS

Animals. Nine 11-day-old rats (25–30 g.) from two litters were each given 0·25 ml. of 0·9% (w/v) sodium chloride solution containing 5 μc of [1-^{14}C]glycine by intraperitoneal injection. Six female adult rats (320–360 g.) were also given an intraperitoneal injection of 16·6 μc of [1-^{14}C]glycine in 0·5 ml. of 0·9% (w/v) sodium chloride solution. Animals were killed at intervals up to 250 days after the injection. Blood was withdrawn from the heart. Brain and spinal cord, sciatic nerve, tail tendon and the quadriceps femoris muscle were removed, washed in saline, dried with filter paper and weighed.

Extraction of nervous tissue

Nervous tissue was homogenized in 20 vol. of chloroform–methanol (2:1, v/v) and the extract filtered. The extract was then washed with 0·2 vol. of water as described by Folch, Lees & Sloane-Stanley (1957). The two phases were allowed to separate, a portion of the upper phase was dried, and radioactivity was determined either on a planchet, as for blood (see below), or after dispersing in xylene plus phosphor, by scintillation counting. Traces of radioactivity remained detectable in the upper phase up to 9 days after injection, so that in rats killed within this time the lower phase, which contained the total lipid and proteolipid, was washed a second time with an equal volume of synthetic upper phase (Folch *et al.* 1957).

Treatment of muscle, tendon and kidney

Muscle protein. Approx. 1 g. of muscle was washed in 0·9% sodium chloride and was dispersed in 20 ml. of 5% (w/v) trichloroacetic acid with the aid of a tissue blender. The precipitated protein was centrifuged down and the procedure repeated until the supernatant was free of radioactivity. The precipitate was then washed with 10 ml. of ethanol and twice extracted with 20 ml. of ethanol–ether (3:2, v/v) at room temperature for 30 min., washed with 10 ml. of diethyl ether and dried *in vacuo*. Some of the dried precipitated protein was macerated with 0·1 N-NaOH for 24 hr., and the alkali-insoluble residue was washed with water and ethanol and then dried *in vacuo* (Lowry, Gilligan & Katersky, 1941). Hydroxyproline estimations indicated that this insoluble residue contained most of the muscle collagen.

Tendon. Tail tendon was washed in 0·9% sodium chloride and cut finely with scissors. The tendon was then treated with 5% (w/v) trichloroacetic acid, washed and dried as described for muscle protein.

Kidney. One kidney was removed, washed free of blood and dried on a filter paper. The kidney was dispersed in 20 vol. of chloroform–methanol (2:1, v/v) and the extract filtered. The residue was washed with 10 ml. of ethanol and then suspended in 25 ml. of water. After standing for 30 min. at room temperature, the suspension was filtered and the residue washed with 10 ml. of ethanol and dried *in vacuo*.

Measurement of radioactivity

Estimation of radioactivity in blood. Blood (0·1 ml.) and plasma (0·1 ml.) were allowed to dry on planchets (1·5 cm. diam.) and radioactivity was determined in a thin-window (mica) Geiger–Müller counter. To follow the persistence of the radioactive glycine in the blood, 0·5 ml. of plasma was also treated with 0·1 ml. of 50% (w/v) trichloroacetic acid and, after centrifuging, 0·1 ml. of protein-free supernatant was dried on a planchet and radioactivity determined.

Scintillation counting. Radioactivity was measured by liquid scintillation counting with the Ekco N612 counter operating at room temperature (Davison, Morgan, Wajda & Payling Wright, 1959; Stitch, 1959). Xylene (May and Baker Ltd., reagent grade) was employed as solvent with 2:5-diphenyloxazole (0·3%, w/v) as scintillator solute. Poly[^{14}C]methyl methacrylate standards of known radioactivity and background samples were counted at intervals throughout the day. There was no indication of instrument drift. Most samples were counted for a total of 10–15 min., but with a few preparations, the radioactivity of which was less than twice background activity (30 counts/min.), counting a sample and background was continued for 60 min.

Determination of radioactivity in total lipid plus proteolipid fraction. The background count of a scintillation-counting cell containing 20 ml. of xylene plus phosphor was determined. The xylene mixture was then poured into a beaker and a suitable volume of the initial chloroform–methanol extract [extract (D), Fig. 1] transferred to the counting cell. The solvent (chloroform–methanol) was removed by passing a stream of nitrogen into the pot. A few drops of methanol were added towards the end of the evaporation to prevent the formation of two phases. Final drying was effected on a water bath at 60°.

After cooling, the 20 ml. of xylene plus phosphor that had been used for the background count was returned to the pot to take up the dried lower-phase deposit, and radioactivity was determined. Traces of insoluble protein in some samples did not cause any loss of radioactivity.

Determination of radioactivity of protein-free lipid. The remaining chloroform–methanol extract was then mixed with an equal volume of synthetic upper phase containing citrate (G. R. Webster & J. Folch, in preparation). After 30 min. insoluble strands of protein separated at the interface between the two phases (stage 4, Fig. 1). This protein, derived from the proteolipid, was removed. No radioactivity was detected in the citrate-containing upper phase after decomposition of proteolipid. A suitable volume of the protein-free lower phase (E) was dried in a flask, and the lipids were dissolved in 20 ml. of xylene plus phosphor. The lipid solution was then transferred to a scintillation counting cell in which background count had already been determined. Radioactivity was measured by scintillation counting (Davison, Morgan, Wajda & Payling Wright, 1959). It was found unnecessary at this stage to wash the lower phase again with synthetic upper phase.

Determination of radioactivity of protein from proteolipid of central nervous system. The material (F), separated from the lower phase after treatment with upper phase containing citrate, was washed with chloroform–methanol (2:1, v/v) and the washings were added to the lower phase. About 2 ml. of ethanol was added to the protein residue, which was then washed with 10 ml. of water. The residue was finally treated with about 5 ml. of acetone and dried in a vacuum desiccator. Although the dry denatured protein residue was ground in a mortar it proved very difficult to produce a suitably fine powder. Particles were mixed with 10 ml. of scintillating gel NE 210 [Nuclear Enterprises

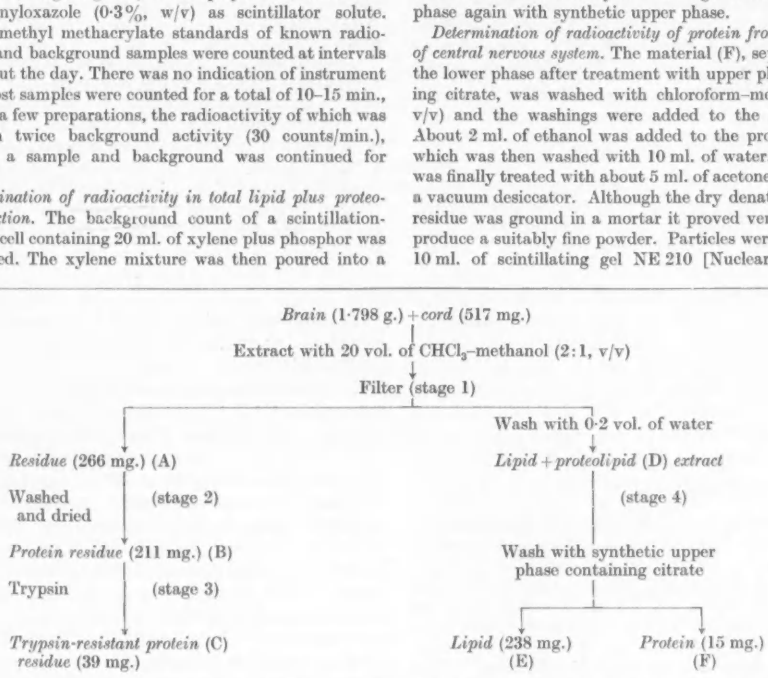
Brain (1·798 g.) + cord (517 mg.)
Extract with 20 vol. of $CHCl_3$–methanol (2:1, v/v)
Filter (stage 1)
Wash with 0·2 vol. of water
Residue (266 mg.) (A)
Lipid + proteolipid (D) extract
Washed and dried
(stage 2)
(stage 4)
Protein residue (211 mg.) (B)
Wash with synthetic upper phase containing citrate
Trypsin
(stage 3)
Trypsin-resistant protein residue (39 mg.) (C)
Lipid (238 mg.) (E)
Protein (15 mg.) (F)

Fig. 1. Treatment of tissue of central nervous system. This figure illustrates the procedures adopted for the separation of nervous tissue. In this experiment wet weights of adult rat brain and spinal cord are given, all other figures are dry weights.

(G.B.) Ltd., Sighthill, Edinburgh 11] in a sample pot and while stirring, the mixture was allowed to gel by warming on a water bath. Radioactivity was determined by scintillation counting.

Radioactivity of brain and spinal cord residues after chloroform–methanol extraction. Residues (A) (Fig. 1), after extraction of lipid, were exhaustively washed and dried as described by LeBaron & Folch (1956). Radioactivity in this dried powder (B) (Fig. 1) was determined in a thin-window Geiger–Müller counter by an infinite-thickness technique and also by counting the radioactivity of a suspension in gel NE 210 with the scintillation counter. The dried powder (50 mg.) was treated with trypsin solution (stage 3, Fig. 1) for a fortnight (LeBaron & Folch, 1956) and then washed with dilute acid, water and acetone before drying. The radioactivity of a suspension of the dried trypsin-resistant protein fraction was determined in gel NE 210.

Validity of the scintillation-counting methods

Since a number of different procedures have been applied to the lipid extract in order to decompose proteolipid, it was necessary to ascertain whether or not such treatment interfered in the determination of radioactivity by scintillation counting. For this purpose an internal standard of ^{14}C-labelled glyceride was added to 20 ml. of xylene plus phosphor containing (1) lipid + proteolipid, (2) washed lipid extract, (3) citrate-treated lipid extract. There was no evidence of any quenching in any of the three preparations.

Protein residues. These were counted as a suspension in a scintillation gel. The validity of this method was investigated in several ways.

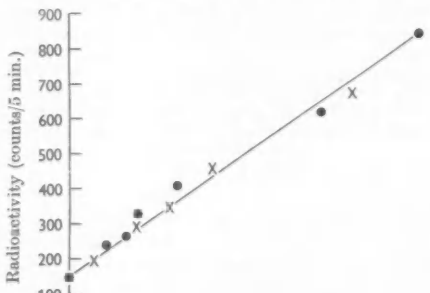

Fig. 2. Radioactivity of ^{14}C-labelled protein suspended in scintillation gel. Different amounts of radioactive protein residue from the central nervous system of an adult rat injected with [1-^{14}C]glycine 15 days earlier have been suspended in scintillation gel. In the first experiment (●) unlabelled protein powder was added to each sample pot to make the total protein content up to 50 mg. and radioactivity was determined by scintillation counting. In the second experiment (×) different amounts of radioactive protein (3·4–50 mg.) were weighed into each sample pot and radioactivity was measured without dilution.

(i) Internal quenching. There was no loss of radioactivity when an internal ^{14}C standard was added to a suspension of 50 mg. of protein powder.

(ii) Particle size. Radioactive protein was powdered in a mortar and some of the powder was passed through a fine sieve (200 mesh). There was only a slight gain (3·5%) of radioactivity in 50 mg. of fine compared to the unsieved powder.

(iii) Sample size. Different amounts up to 50 mg. of radioactive protein powder diluted with non-radioactive protein to give 50 mg. of protein gave a proportionate increase in radioactivity (Fig. 2). Different amounts up to 50 mg. of undiluted radioactive protein powder gave similar increases in radioactivity.

(iv) Comparison with Geiger–Müller counting. Finally the reliability of the technique was checked by comparing the proportion of radioactivity found with the Geiger-Müller counter with that with the scintillating counter (Table 2). The rates of loss of radioactivity are comparable but scintillation counting, operating with a much greater efficiency, gives more consistent results.

Analytical methods

Protein. This was determined with bovine albumin as a standard by the biuret method (Robinson & Hogden, 1940) as modified by Aldridge (1957). Amino acids from protein hydrolysates were separated by two-dimensional chromatography on buffered paper with butanol–acetic acid–water (4:1:5, by vol.) and *m*-cresol–phenol (1:1, v/v) as solvents (Levy & Chung, 1953).

Nitrogen and phosphorus. These were determined as described by Davison & Wajda (1959).

Hydroxyproline. Hydroxyproline was assayed by the method of Neuman & Logan (1950) on protein residues hydrolysed in 1 ml. of 6N-HCl in a sealed tube for 6 hr. at 110°. Collagen contains 14·5% of hydroxyproline (Neuberger, 1955), and since this amino acid is not present in other groups of protein it was possible to calculate the approximate collagen content of a specimen.

RESULTS

In this work the incorporation and persistence of radioactivity in the proteins of the central and peripheral nervous system, muscle and tendon have been followed for long periods after intraperitoneal injection of [1-^{14}C]glycine into neonatal and mature groups of rats. In an earlier experiment in which ^{32}P was used in metabolic studies of cerebral phospholipids (Davison & Dobbing, 1960*a*) ^{32}P-labelled precursors remained in the blood for long periods. Since such persistence of radioactive precursor makes interpretation of long-term metabolic studies difficult, levels of radioactivity in whole blood and plasma were followed in all animals injected with labelled glycine. To exclude the possibility that this radioactivity is due to persistence of non-protein ^{14}C, plasma was treated with trichloroacetic acid and radioactivity was determined in the protein-free supernatant obtained after centrifuging. There was a progressive

Table 1. *Analysis of protein samples*

Methods for the preparation of the dried protein samples and for their analysis are given in the text. Collagen figures are derived from the amount of hydroxyproline × 6·9. Samples analysed were obtained from young adult rats.

Sample	Protein (%)	Nitrogen (%)	Collagen (%)	Phosphorus (%)
Brain and cord residue	100·2	12	1·5	0·4
Proteolipid protein	—	—	0	0·4
Sciatic nerve residue	—	7·2	11	0·4
Muscle residue	97·5	14	3·8	0·1
Tendon	88	13	100	0·02
Kidney residue	97	—	—	—

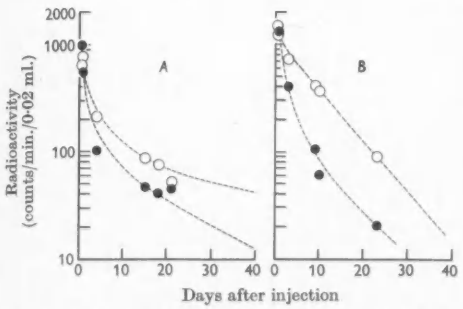

Fig. 3. Levels of radioactivity in plasma and whole blood of adult rats (*A*) and neonatal rats (*B*) at various intervals after the administration of radioactive glycine. Radioactivity in plasma (●) and whole blood (○) was determined by Geiger–Müller counting as described in the text. The extrapolation of the curves beyond the last readings shown is based on further measurements made at 48 and 72 days for young rats and 42 and 72 days for adults.

loss of radioactivity from the blood of young and adult rats, the rate being greater for plasma than for whole blood (Fig. 3). No radioactivity was detected in the protein-free plasma extract 8 days after injection in the neonatal animals, and only 3 % of the plasma radioactivity was in this form 19 hr. after injection. In the adult animals protein-free plasma extract was free of detectable radioactivity 24 hr. after injection of the glycine. These results indicate that circulating radioactive precursors rapidly disappear from the blood (see also Greenberg & Winnick, 1948; Henriques, Henriques & Neuberger, 1955), and, therefore, the present investigation is not complicated as was the ^{32}P study by this persistence.

Proteins of the central nervous system. Rats were killed at intervals after injection with labelled glycine, and the brain and spinal cord extracted with chloroform–methanol (2:1, v/v). This initial treatment (stage 1) separated lipid and most of the proteolipid of the central nervous system from an insoluble residue [residue (A), Fig. 1]. After removal of a sample of the washed chloroform–methanol extract, the remainder was mixed with a synthetic upper phase containing potassium citrate (G. R. Webster & J. Folch, in preparation) to dissociate the proteolipid (stage 4, Fig. 1). The proteolipid protein (F) obtained by this latter treatment was washed with different solvents (see above) and analysed for nitrogen and phosphorus (Table 1). A sample was also refluxed with 6N-hydrochloric acid for 6 hr. and the hydrolysate separated by paper chromatography; it contained at least 18 different amino acids, confirming that the substance was a protein.

Analysis of the chloroform–methanol–insoluble residue from the brain and cord indicated that this consisted almost entirely of protein. Traces of carbohydrate and phosphorus and the equivalent of about 1·5 % of collagen were also present in the protein residue (Table 1). Some of the chloroform–methanol-insoluble residue was incubated with trypsin (LeBaron & Folch, 1956) and the trypsin-resistant protein residue prepared.

Persistence of radioactivity in the proteins of the central nervous system of the rat. The ^{14}C content of proteolipid protein was determined by two techniques. The first and more accurate method (method 1) depended on the difference between the radioactivity of an extract containing chloroform–methanol–soluble lipid plus proteolipid and the radioactivity of the same extract after removal of proteolipid protein. In some cases radioactivity of the separated proteolipid protein was determined directly (method 2). Difficulties in producing a fine powder suitable for scintillation counting account for any differences in radioactivity content found by the two methods (Tables 2 and 3).

The proteolipid protein that was obtained from rats, which had been injected with [1-^{14}C]glycine when 11 days old, retained its radioactivity for at least 250 days (Table 2 and Fig. 4). After injection of labelled glycine into adult rats much less ^{14}C was incorporated into the proteolipid protein of the central nervous system. Thus a day after injection the corrected specific activities of proteolipid protein ($\mu\mu$c/mg. of protein) were 373 and 2·5 for neonatal and adult rats respectively. Further, the results suggest that the labelled proteolipid protein

Table 2. *Radioactivity of proteins of the central nervous system at intervals after injection of* [1-^{14}C]*glycine into* 11*-day-old rats*

Rats previously injected with 5 μc of [1-^{14}C]glycine were killed at intervals up to 250 days. The preparation of the various samples is described in the text. Radioactivity of the 'protein' residue counted at infinite thickness by thin end-window Geiger–Müller counter is expressed in arbitrary units equivalent to counts/min. of sample. Radioactivity of other protein fractions has been determined by scintillation counting as described in the text. Figures for radioactivity should be multiplied by 0·6 to give the equivalent of a dose of 10 μc of [1-^{14}C]glycine/100 g. body wt.

				Radioactivity of total central nervous system					
				Whole protein residue less proteolipid protein [residue (A)]				Proteolipid protein	
Time after injection	Body wt. when killed (g.)	Wet wt. of brain and cord (g.)	Wt. of washed chloroform–methanol-insoluble residue (A) (mg.)	Determined by Geiger–Müller counting (counts/min.)	Whole untreated residue determined by scintillation counting ($\mu\mu$c)	Whole alkali-stable residue ($\mu\mu$c)	Whole trypsin-resistant protein residue ($\mu\mu$c)	Determined by difference in radioactivity of lipid extracts (D) and (E), method 1 ($\mu\mu$c)	Determined directly by counting suspension (F) in gel, method 2 ($\mu\mu$c)
2 hr.	25	1·19	200	204	27 800	—	—	—	—
18	30	1·21	160	200	17 000	—	—	—	—
19	30	1·22	140	178	11 400	—	1750	620	—
3 days	28	1·37	80*	102	8 100	640	1060	470	—
9	55	1·48	127	—	10 700	—	1200	—	—
10	52	1·76	144	110	—	—	780	350	155
23	134	2·0	208	71	5 320	—	780	450	—
42	168	2·16	291	47	2 380	—	444	270	290
72	380	2·41	223	19	1 030	—	185	220	400
126	390	2·54	251	15	670	160	122	405	—
189	280	2·71	268	6	300	160	64	260	280
250	258	2·59	264	3	228	127	—	290	190

* This result is low owing to poor recovery.

Table 3. *Radioactivity of proteins of the central nervous system at intervals after injection of* [1-^{14}C]*glycine into mature rats*

Six adult female rats of about 350 g. were given 16·6 μc of [1-^{14}C]glycine by intraperitoneal injection. For details of determination of radioactivity see Table 2 and text. Figures for radioactivity should be multiplied by 2·1 to give the equivalent of a dose of 10 μc of [1-^{14}C]glycine/100 g. body wt.

			Radioactivity of total central nervous system					
			Whole protein residue less proteolipid protein [residue (A)]				Proteolipid protein	
Time after injection (days)	Wet wt. of brain and cord (g.)	Wt. of washed chloroform–methanol-insoluble residue (mg.)	Determined by Geiger–Müller counting (counts/min.)	Whole untreated residue determined by scintillation counting ($\mu\mu$c)	Whole alkali-stable residue ($\mu\mu$c)	Whole trypsin-resistant protein residue (C) ($\mu\mu$c)	Determined by difference in radioactivity of lipid extracts (D) and (E), method 1 ($\mu\mu$c)	Determined directly by counting suspension (F) in gel, method 2 ($\mu\mu$c)
1	2·98	309	43	3520	—	60	—	29
4	2·63	211	18	1770	—	232	32	23
18	2·76	246	14	1120	—	210	20	34
49	2·87	244	4	590	18	57	78	44
190	2·69	259	1	78	0	0	5	4
197	3·15	280	2	50	0·1	35	12	8

of the central nervous system in adult rats undergoes slow turnover (Table 3). Clouet & Richter (1959) found that there was relatively slow incorporation of ^{35}S into the cerebral proteolipid protein of 28-day-old rats.

The major proportion of brain and spinal cord proteins (excluding proteolipid protein and collagen) are in a state of dynamic metabolism. After injection of [1-^{14}C]glycine into both neonatal and adult groups of rats relatively large amounts of ^{14}C are incorporated into the central-nervous-system proteins [residue (B), Fig. 1]. The rate of loss of labelled protein from the central nervous system of both groups is exponential (see Fig. 4 and Tables 2 and 3) with a half-life of about 22 days. Some conservation of radioactivity in the protein residue of rats, which had been injected with [1-^{14}C]glycine when 11 days old, was associated with the alkali-insoluble protein, and thus this apparent persistence of about 1% of the initial radioactivity can be ascribed to retention of ^{14}C in collagen of the central nervous system.

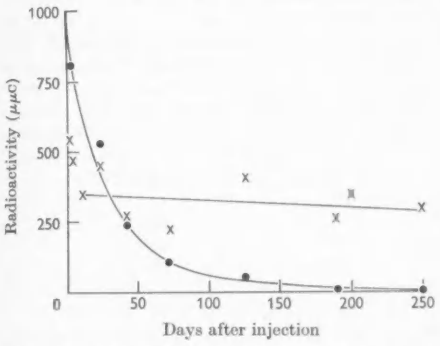

Fig. 4. Levels of radioactivity in proteins of the central nervous system of rats at various intervals after the administration of [1-^{14}C]glycine. Radioactivity of total central-nervous-system protein other than proteolipid protein is for convenience divided by 10 (●). Persistence of radioactivity in whole brain and cord proteolipid protein (×) is indicated by the straight line calculated by the method of least squares. The line includes values from 10 to 250 days after injection of labelled glycine. Results are expressed in terms of a dose of 5 μc/[1-^{14}C]glycine injected into 11-day-old rats of a mean body weight of 30 g.

Persistence of radioactivity in the proteins of the peripheral nervous system. Radioactivity of chloroform–methanol-insoluble protein of sciatic nerve has been determined, at intervals after injection of neonatal and adult rats, by scintillation counting of the dry protein powder in suspension. Since it is not practicable to dissect out whole sciatic nerve in a reproducible way for different animals, particularly young rats, results for nerve are recorded as specific activities. The specific activity is expressed as radioactivity/mg. of dried protein residue. Specific activities are further corrected to a dose of 10 μc/100 g. body wt. (to facilitate comparison between neonatal and adult groups of rats), and the results for the neonatal group of rats are also corrected for the dilution effect of growth. This latter correction is based on the assumption that increase in nerve protein parallels increase in body weight. Such an assumption seems reasonable as the increases in wet weight of sciatic nerve recovered are proportional to body weight during development.

If the results obtained for the neonatal and adult rats are compared two important differences emerge. First, much less radioactive glycine is

Table 4. *Persistence of radioactivity in the protein residue from the sciatic nerve of young and adult groups of rats at intervals after an injection of* [1-^{14}C]*glycine*

Methods for the preparation and determination of sample radioactivity are given in the text. Specific activity is given as radioactivity μμc/mg. of protein residue corrected for a dose of 10 μc of [1-^{14}C]glycine/100 g. body wt. Specific activities for young rats are also corrected for the dilution effect of growth.

Rats injected with 5 μc of [1-^{14}C]glycine when 11 days old				Rats injected with 16·6 μc of [1-^{14}C]glycine when adult			
Time after injection (days)	Wt. of sciatic nerve (mg.)	Wt. of chloroform–methanol-insoluble protein residue (mg.)	Specific activity corrected for growth and dose	Time after injection (days)	Wt. of sciatic nerve (mg.)	Wt. of chloroform–methanol-insoluble residue (mg.)	Specific activity corrected for dose
1	16	1·1	265	1	134	18·3	30
42	60	2	87	4	115	11	5·7
72	131	11	254	18	139	15·7	12·0 (1·6)*
126	166	18	140	49	123	7·4	1·9
189	92	12	210 (63)*	190	115	17·8	2·3 (0·4)*
250	90	10	67 (46)*	197	140	14·4	1·3 (0·6)*

* Radioactivity of alkali-insoluble protein residue of sciatic nerve.

incorporated into the nerve proteins of adult compared to the 11-day-old rats. Secondly, the specific activity of sciatic-nerve protein in the mature series of rats declines during the period studied, and only about 7 % of the original activity remains 190–197 days after injection. In those rats injected with [1-^{14}C]glycine when 11 days old, 25 % of the original radioactivity remains 250 days after injection (Table 4).

Persistence of radioactivity in the proteins of muscle and tendon. Radioactivity of muscle and tendon protein was determined by scintillation counting of a suspension in a gel. Since it is impracticable to express the radioactivity as total body muscle or tendon protein activity the results are shown as specific activities corrected for a dose of [1-^{14}C]glycine of 10 μc/100 g. body wt. and for the dilution effect of growth in the developing rats (Table 5). This later correction is based on the assumption that accumulation of unlabelled protein parallels increase of body weight (Neuberger, Perrone & Slack, 1951). Even if no corrections are applied the same general picture emerges. Radioactivity was also determined in the alkali-insoluble muscle and tendon residues. The specific activities were calculated in terms of the weight of original material taken before alkali digestion. Table 5 shows that there is rapid incorporation of glycine into muscle protein and particularly into the tendon of the developing rats. There is loss of radioactivity from both proteins up to about 23 days after injection of the precursor into neonatal rats, after that the level of radioactivity remains with little change until the end of the experiment (250 days). Almost all the radioactivity incorporated into the tendon is located in alkali-stable protein, which is largely collagen. In the muscle protein the [1-^{14}C]glycine is incorporated into both alkali-soluble and alkali-insoluble protein and the persisting radioactivity remains in both fractions for at least 250 days.

Much less radioactivity is incorporated into muscle and tendon protein of the adult rats. Further, a high proportion of this radioactivity is found in the alkali-soluble muscle protein (Table 6). The radioactivity is lost only slowly from the labelled muscle and tendon protein.

Table 5. *Radioactivity of tendon and muscle proteins at intervals after injection of* [1-^{14}C]*glycine into* 11*-day-old rats*

Methods for extraction and for the determination of radioactivity by scintillation counting are described in the text. The results are given as specific activities corrected for the dilution effect of growth and for a dose of 10 μc of [1-^{14}C]glycine/100 g. body wt. All specific activities are recorded as radioactivity $\mu\mu$c/mg. of dry unhydrolysed protein.

	Radioactivity in collagen		Radioactivity in muscle protein	
Time after injection of 5 μc of [1-^{14}C]glycine	Protein residue specific activity	Alkali-stable residue specific activity	Protein residue specific activity	Alkali-stable residue specific activity
19 hr.	104	58	17	12
3 days	220	185	65	14
23	71	100	56	12
42	53	54	35	5
72	20	27	35	10
126	37	27	36	10
189	37	48	36	13
250	52	43	13	6

Table 6. *Radioactivity of tendon and muscle proteins at intervals after injection of* [1-^{14}C]*glycine into mature rats*

For details see Table 5.

	Radioactivity in collagen		Radioactivity in muscle protein	
Time after injection of 16·6 μc of [1-^{14}C]glycine (days)	Protein residue specific activity	Alkali-stable residue specific activity	Protein residue specific activity	Alkali-stable residue specific activity
1	1·93	1·6	5·5	0·6
4	1·64	—	9·6	7·4
18	0·73	—	17·0	4·9
49	0·45	—	8·7	3·3
190	0·26	0·38	0·75	0
197	0·15	0·12	2·1	0·1

Fate of radioactivity incorporated into kidney proteins

Radioactivity was also determined in the kidney proteins at intervals after the injection of [1-^{14}C]-glycine into 11-day-old and adult rats (Table 7). The results in Table 7 show the total radioactivity of the protein from one kidney of each rat. After an initial period (4–10 days) in both neonatal and adult groups of rats there is a steady loss of ^{14}C from the kidney proteins. The small amount of radioactivity found at the end of the experiment in the proteins of the developing rats is almost entirely restricted to the alkali-insoluble protein residue (collagen) from the kidney.

DISCUSSION

The classical studies of Schoenheimer (1946) led to the discovery of the dynamic state of body proteins and other constituents. Subsequently many workers have confirmed and extended Schoenheimer's original concept of rapid protein metabolism. Thus it has been shown in experiments *in vivo* that labelled amino acids can be readily incorporated into brain and other body proteins

Table 7. *Radioactivity of kidney lipid-free residue at intervals after injection of* [1-^{14}C]*glycine into neonatal and mature rats*

Kidney was extracted with chloroform–methanol (2:1, v/v) and after filtration the residue was successively washed with ethanol, water and ethanol. Radioactivity of the total dried residue (about 97% of protein) was determined by scintillation counting, and the results are corrected to the equivalent of a dose of 10 μc of [1-^{14}C]-glycine/100 g. body wt. Neonatal and mature rats were injected with 5 and 16·6 μc of [1-^{14}C]glycine respectively.

Neonatal rats			Mature rats		
Time after injection (days)	Dry wt. of residue (mg.)	Radioactivity ($\mu\mu$c)	Time after injection (days)	Dry wt. of residue (mg.)	Radioactivity ($\mu\mu$c)
1	18·1	2840	1	161·8	10 700
1	30·7	1270	4	125·5	13 700
3	11·3	1140	18	245·4	1 700
9	31·2	1305	49	179·6	597
10	11·2	1650	190	222·2	258
23	46·4	625	197	260·9	354
42	132·7	630	—	—	—
72	211·4	150	—	—	—
126	178·2	130	—	—	—
250	118·4	77	—	—	—

(Greenberg & Winnick, 1948; Vladimirov & Urinson, 1957; Kravchinsky & Silich, 1957; Furst, Lajtha & Waelsch, 1958; Clouet & Richter, 1959; Lajtha, Berl & Waelsch, 1959). Although it is recognized that brain proteins are not homogeneous and the range of turnover rates of brain proteins is very wide and possibly includes less active or completely inactive fractions (Clouet & Richter, 1959; Lajtha, 1959), these short-term studies were taken to indicate that adult rat-brain and mouse-brain proteins as a whole have a mean half life of between 14 and 27 days.

Although very slow turnover rates have been reported for part of muscle protein (Neuberger *et al.* 1951; Thompson & Ballou, 1954) and for collagen (Neuberger & Slack, 1953) it does not appear that any attention has been paid to studying the retention of labelled neural proteins for long periods. Neither has there been any systematic comparison between long-term protein metabolism in young and adult animals.

The present work indicates that neural and muscle proteins are composed of groups of both metabolically active and inactive protein. The definition of metabolically inactive or stable proteins used in this present work applies to an experiment lasting only 250 days. It is therefore possible that very slow turnover may occur during the lifetime of the rat or equally that there may be complete metabolic inertness of certain protein fractions. Another possibility that must be considered is that internal exchange and re-utilization of radioactive amino acid occurs *in situ*; however, if this was happening it is probable that a slow loss of radioactivity would become apparent. Further, if recycling of degradation products of radioactive protein was occurring such a process must be restricted to the proteolipid proteins, for there was a continuous loss of radioactivity from other proteins of the central nervous system.

Protein metabolism of the central nervous system. It has been shown that metabolism of proteins, like that of lipids, can be divided into metabolically active and inactive pools. Those proteins which are metabolically active comprise the major part of the proteins of the central nervous system and include the trypsin-resistant protein residue part of which probably comprises the chemically inert neurokeratin (LeBaron & Folch, 1956). The mean turnover rates of these groups of proteins are indicated in Table 8 together with those obtained by other authors. In the present work the turnover rates for protein residues of both young and adult groups of rats are the same and in reasonable agreement with the half-lives reported by other authors for adult rats and mice. In these latter experiments [^{35}S]methionine, [^{14}C]leucine or lysine was injected into animals and the half-life was calculated from the degree of incorporation into brain proteins. The faster turnover rates obtained (Lajtha, 1959; Lajtha, Furst, Gerstein & Waelsch, 1957) for very young mice may be due, as suggested by the authors, to predominant incorporation into rapidly metabolizing brain-protein fractions. Another factor influencing the rate of incorporation of amino acids into brain protein is that in young animals structural, relatively inert, proteins are being synthesized and that turnover rates determined by extrapolation will thereby be artificially increased. These limitations of the short-term metabolism experiments are, however, clearly recognized by Clouet & Richter (1959) and by Lajtha (1959).

In those animals injected with [1-^{14}C]glycine when 11 days old, there is a persistence of radioactive proteolipid protein lasting for at least

Table 8. *Mean turnover rates of labelled proteins of the central nervous system*

Mean turnover rates of whole brain and cord (1) were obtained directly from the biological decay curve. Other mean turnover rates are for brain proteins and were calculated from the incorporation curves obtained in short-term experiments.

Animal	Age (days)	Amino acid injected	Duration of expt.	Protein preparation	Mean half-life (days)	Reference
Rat	11	[1-^{14}C]Glycine	250 days	Whole protein central nervous system residue	22	1
				Trypsin-resistant residue	22	1
Rat	Adult	[1-^{14}C]Glycine	250 days	Whole protein central nervous system residue	22	1
				Trypsin-resistant residue	22	1
Rat	Young (36–77 g.)	[^{35}S]Methionine	20–60 min.	Mixed brain proteins	14	2
Mouse	Newborn	[^{14}C]Leucine	60 min.	Mixed brain proteins	4·1	3
Mouse	Adult	[^{14}C]Leucine	60 min.	Mixed brain proteins	27	3
Mouse	8	[^{14}C]Lysine	45 min.	Mixed brain proteins	2·4	4
Mouse	Adult	[^{14}C]Lysine	60 min.	Mixed brain proteins	15·2	4

1, This paper; 2, Gaitonde & Richter (1956); 3, Lajtha (1959); 4, Lajtha, Furst, Gerstein & Waelsch (1957).

250 days. This persistence indicates a quite remarkable degree of metabolic inertness compared with that of other brain and cord proteins. In addition, a relatively small amount of radioactivity persisting in the protein residue of the central nervous system has been shown to be probably due to the retention of ^{14}C in the collagen present in the organ.

In contrast with these findings in the young rats, much less radioactive glycine is incorporated into the relatively larger proteolipid-protein content of the adult rat brain and cord. Such radioactivity as is incorporated undergoes slow turnover and may well be associated with non-permanent cell or particle membranes. Similarly, there is little retention of radioactivity in other proteins from the central nervous system of adult rats injected with [1-^{14}C]glycine. These results support the hypothesis that proteolipid protein is an integral part of the myelin sheath and, if inert, once labelled during development the radioactivity would persist throughout life in the healthy normal animal. It would also follow that if proteolipid protein was predominantly inert there would be a failure to incorporate and retain radioactive glycine in this protein in the adult animal.

Protein metabolism of the peripheral nervous system. The results shown in Table 4 indicate that a larger fraction of nerve-protein residue retains the incorporated radioactivity than is retained in the protein residue of the central nervous system. This persistent radioactivity is not wholly due to the labelling of metabolically inert collagen or elastin present in the nerve. Peripheral nerve differs from the central nervous system in that only traces of proteolipid soluble in chloroform–methanol can be extracted from nerve (Folch *et al.* 1958; Finean, Hawthorne & Patterson, 1957). It is therefore probable that the protein residue obtained from the sciatic nerve contains relatively large quantities of myelin-sheath protein and that it is this protein fraction which accounts for the conservation of stable ^{14}C in the residue.

In the adult rat loss of radioactivity from the residue of sciatic nerve insoluble in chloroform–methanol is comparable to the loss of radioactivity from the protein residue of the central nervous system. This difference in the behaviour of nerve protein in the young and adult group of animals suggests that in the adult only non-structural proteins incorporate radioactive glycine and that the structural proteins remain metabolically inert and inaccessible to the precursor.

Protein metabolism of muscle and collagen. It was first recognized by Sprinson & Rittenburg (1949) that although proteins of plasma and some of the internal organs undergo rapid metabolism (see Greenberg & Winnick, 1948, and this paper) other proteins of muscle, skin, etc. are relatively inert metabolically. Direct experimental evidence for this concept was reported by Neuberger and his associates (Neuberger, 1955; Henriques *et al.* 1955) and by Thompson & Ballou (1954, 1956). The present experiments differ in some respects from those of these earlier workers. Thus not only have more animals been used in experiments over a considerably extended period but some attempt has also been made to divide the protein into alkali-stable ('collagen') and alkali-soluble fractions.

Neuberger *et al.* (1951) showed that much less [^{14}C]glycine was incorporated into collagen of old

compared with young rats and that there was retention of the radioactivity incorporated into the collagen of the young animals. These findings were confirmed in the present investigation. It also appears that collagen, like other body constituents, can be divided into metabolically active and inactive groups. Thus in young animals there was turnover of incorporated radioactive carbon until 42 days after injection. After that, between 42 and 250 days, there was almost complete retention of radioactivity. Similarly, [1-^{14}C]glycine was incorporated into alkali-soluble and alkali-insoluble (collagen) muscle proteins of neonatal rats. There was little turnover of labelled muscle collagen, but other muscle proteins lost about half the incorporated ^{14}C up to 42 days after injection. From 42 to 250 days after injection there was a retention of radioactivity. These findings suggest that part of the actomyosin may be metabolically inert.

After injection of labelled glycine into adult rats a relatively small amount of ^{14}C was incorporated into collagen and muscle proteins. There was slow turnover of the labelled collagen and no indication of the metabolic stability of the major part of the collagen, which can only be demonstrated by introducing radioactive glycine into developing rats. Up to 18 days after injection there was an increase in radioactivity incorporated into the muscle protein of adult rats. Recently Dreyfus, Kruh & Schapira (1960) have demonstrated a similar retention of ^{14}C-labelled myosin in adult rats injected with glycine uniformly labelled with ^{14}C. As these authors suggest, the persistence of radioactivity in the myosin may only be apparent, for they demonstrated that free labelled glycine remains in muscle for several weeks after injection. Thus it is probable that the increase in radioactivity of muscle protein of adult rats up to 18 days after injection, reported in the present paper, was due to continued synthesis of new labelled protein. This possibility is supported by the finding of Dreyfus *et al.* (1960) that there is an immediate and progressive loss of radioactivity from myosin in rats fed on a high-protein diet when the free labelled glycine of muscle would be expected to be rapidly replaced by non-radioactive amino acid. In the present study the fate of radioactive muscle protein was followed for a period up to 196 days after the initial injection. From 18 days after injection to the end of the experiment, there was a slow loss of ^{14}C from the muscle protein. Unfortunately the experiments of Dreyfus *et al.* (1960), suggesting that adult rat myosin is relatively inert, were continued for only 55 days and slow turnover of myosin was only observed by them in rats fed a high-protein diet. Metabolic stability of part of the muscle protein can, however, be demonstrated by incorporating [1-^{14}C]glycine into developing rats (see above).

The present findings therefore fit in with the general hypothesis that body constituents can be divided into metabolically active and metabolically inert (structural) compartments. Wholly metabolically active constituents are typified by the plasma or kidney proteins or by the lipids of some of the internal organs. At the other extreme are deoxyribonucleic acid (Hecht & Potter, 1958), haemoglobin (Shemin & Rittenberg, 1946; Muir, Neuberger & Perrone, 1952) and some of the brain lipids (Davison & Dobbing, 1960*a*) which are probably metabolically inert. Other body constituents can be divided into both stable and metabolically active compartments. Thus in the present work a proportion of the muscle and collagen proteins and some of the proteins of the central nervous system have been shown to be stable, the remainder undergoing dynamic metabolism.

SUMMARY

1. After the injection of [1-^{14}C]glycine into neonatal and mature rats it has been shown that proteins of the central and peripheral nervous system, of muscle and tendon can be divided into metabolically active and inactive groups.

2. Metabolic inertness of proteolipid protein of the central nervous system is suggested by the retention of radioactivity in this protein for at least 250 days after injection of [1-^{14}C]glycine into neonatal rats.

3. Relatively little radioactivity is incorporated into the proteolipid protein of adult rats injected with labelled glycine and the radioactive proteolipid protein undergoes slow turnover.

4. Proteins of the central nervous system, other than collagen and proteolipid, in both young and adults have been shown to be metabolically active with turnover rates comparable with those of plasma and kidney proteins.

5. Radioactivity persists for at least 250 days in sciatic nerve collagen and other proteins after injection of 11-day-old rats, but in adult rats nerve proteins undergo dynamic metabolism.

6. Similar metabolic inertness has been observed in some muscle and tendon proteins after injection of neonatal but not mature rats.

7. It is suggested that the metabolic inertness of certain neural, muscle and tendon proteins is associated with stable structural elements such as the myelin sheath, collagen and actomyosin. This metabolic stability can only be demonstrated by incorporation of radioactive isotopes into these proteins during development.

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The Location of Carbohydrases in the Digestive Tract of the Pig

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Little is known about the locations in the mammalian intestine at which hydrolysis of disaccharides and polysaccharides occurs.

In a recent investigation of intestinal digestion and absorption in man, Borgström, Dahlqvist, Lundh & Sjövall (1957) observed that the invertase activity (in unpublished experiments we have demonstrated that the maltase and lactase activities are distributed similarly) of the intestinal contents, which was weak throughout the whole small intestine, was maximal in the lower part of the small intestine (lower jejunum and ileum). In spite of this, the absorption of lactose occurred in the upper part of the small intestine (duodenum and upper jejunum), where no disaccharidase activity could be demonstrated in the intestinal contents (Ammon & Henning, 1956; Borgström *et al.* 1957). The hydrolysis of disaccharides during their absorption is catalysed by enzymes apparently situated inside the cells of the intestinal mucosa (Borgström *et al.* 1957). Little information is, however, available about the relative disaccharidase activity of the mucosa of different parts of the small intestine.

Amylase, in contrast to the disaccharidases, is mainly secreted into the intestinal lumen in the

pancreatic juice. However, preparations from the intestinal mucosa also contain amylase. Intestinal-mucosa preparations from different species of mammals have been reported to contain one further polysaccharidase, namely dextranase (Adrouny, Bloom & Wilhelmi, 1957). It does not seem to be known, however, whether dextranase is present in the pancreas too.

This paper records the carbohydrase activities of homogenates of mucosa from different parts of the small intestine, from the stomach and from the colon, and of a homogenate of pancreatic tissue. The adult pig was selected as an experimental animal.

EXPERIMENTAL

Determination of enzymic activities

Disaccharidase activities. These were measured by the methods described previously (Dahlqvist, 1960*d*). One unit of disaccharidase activity causes 5% of hydrolysis of the particular disaccharide in 2·0 ml. of reaction mixture at 28 mM-substrate concentration in 60 min. at 37°.

Amylase activity. The substrate solution was prepared by dissolving 2·0 g. of soluble starch a.m. Zulkowsky (from Merck A.G., Germany) and 40 mg. of NaCl in 0·05 M-phosphate buffer, pH 6·9 (3·026 g. of KH_2PO_4 and 3·959 g. of $Na_2HPO_4,2H_2O$/l.), to a final volume of 100 ml. Toluene (1 ml.) was added as a preservative and the solution was stored in a refrigerator. The substrate solution was prepared weekly.

For determination of amylase activity, 1·0 ml. of suitably diluted enzyme solution was mixed with 1·0 ml. of substrate solution and immersed in a water bath at 37°. After 60 min. the reaction was interrupted by the addition of 2·0 ml. of dinitrosalicylate reagent (prepared as described by Hostettler, Borel & Deuel, 1951). A blank was prepared with the same composition, in which, however, the 3:5-dinitrosalicylate reagent was added immediately after the mixing of the enzyme and substrate. The tubes were immersed in a boiling-water bath for 10 min. and then chilled for 2 min. with running tap water. After dilution with 20·0 ml. of water the intensity of the red colour produced was measured in a Beckman B spectrophotometer at a wavelength of 530 mμ, in 1 cm. cuvettes. A standard curve was prepared from known solutions of maltose (the tubes containing 0·5–2·0 mg. of maltose monohydrate). One unit of amylase activity causes an increase of reducing power corresponding to 1 mg. of maltose monohydrate in 60 min. during these conditions. If the increase of reducing power does not exceed that of 2 mg. of maltose monohydrate, the enzymic reaction follows zero-order kinetics and the amount of reducing groups liberated is proportional to the amount of enzyme present.

The amylase unit, when defined in this way, is comparable with the unit used for disaccharidase activity (Dahlqvist, 1960*d*). It should not, however, be confused with the amylase unit used by Borgström *et al.* (1957), since in that case the incubation was performed for 3 min. at 25°.

Dextranase activity. The substrate solution for determinations of dextranase activity was prepared by dissolving 2·0 g. of dextran (dextran 40, mol.wt. by light-scattering 41 000, by end-group analysis 26 000, obtained from Pharmacia A.B., Sweden) in 0·1 M-maleate buffer (Gomori, 1955), pH 6·0, to a final volume of 100 ml. Incubation and determination of the degree of hydrolysis were performed in exactly the same way as for determinations of amylase activity. Maltose was used for preparation of the standard curve, since maltose and isomaltose have the same extinction coefficient with the 3:5-dinitrosalicylate reagent. One unit of dextranase activity is the amount of enzyme which causes an increase of reducing power equal to that of 1 mg. of maltose monohydrate in 60 min.

Determination of protein

The method of Lowry, Rosebrough, Farr & Randall (1951) was employed, the modified reagent B introduced by Eggstein & Kreutz (1955) being used. A standard curve was prepared with human serum albumin (kindly supplied by A. B. Kabi, Sweden).

Preparations of homogenates

The stomach, small intestine, upper part of the colon and pancreas of an adult pig were cut out immediately after slaughter and chilled with crushed ice during transport to the laboratory. From pieces of stomach, small intestine and colon the mucosa was scraped off with a glass slide and homogenized in an Ultra-Turrax homogenizer for 2 min. with an equal weight of 0·9% NaCl. A piece of the pancreas was homogenized in the same way. This method has earlier been found suitable for the extraction of glycosidases from hog small-intestinal mucosa (Borgström & Dahlqvist, 1958). After centrifuging in a Wifug laboratory centrifuge for 5 min., the opalescent supernatant was assayed for carbohydrase activities.

RESULTS

There are several ways of expressing the relative carbohydrase activities of the different segments of the intestine. Some authors have expressed the activity per cm.^{2} of intestine (Euler & Svanberg, 1921). Because of the enormous surface area of intestinal villi, as well as their variation in density along the length of the intestine, it seems more logical to compare the activity per gram of mucosa (Cajori, 1935; Heilskov, 1951). Since in the present investigation the homogenates have always been prepared from a mixture of equal weights of mucosa and 0·9% NaCl, the carbohydrase activities can be compared directly when expressed per ml. of homogenate preparations.

The protein content of the homogenates varied (Table 1), however, and therefore in Figs. 1–5 the specific carbohydrase activity (i.e. number of units/mg. of protein) has been used to enable comparison of the relative carbohydrase activities of the different segments of the small intestine. However, the results are similar whether enzyme activity is presented as units/mg. of mucosal protein or units/ml. of mucosal homogenate. The experimental conditions for carbohydrase assay were such that 0·5 unit/ml. of each of the activities investigated could be detected.

To check the completeness of the removal of the mucosa with the glass slide, in one experiment the remaining intestinal wall was also homogenized, and the homogenate was assayed for invertase activity. It was found that 80% of the total amount of invertase originally present in the piece of intestine had been removed with the mucosa.

Stomach. No invertase, maltase, isomaltase, trehalase, lactase, cellobiase or dextranase activity could be detected. The preparation had very weak amylase activity (17 units/ml.; cf. below), which may very well have been caused by contamination with saliva from the gastric contents. The mucosa of the stomach therefore does not seem to contain any carbohydrases.

Small intestine. This had a total length of 16 m. The duodenum had a length of 0·34 m., the jejunum and the ileum had each a length of about 8 m. In the duodenum and jejunum no intestinal contents had to be removed, but in the ileum the contents were removed and the intestine was gently blotted with a piece of cloth before the mucosa was scraped off. The pieces of the small intestine selected are noted in Figs. 1–5. The amount of mucosa obtained from 50 cm. of small intestine varied between 7 and 11 g.

Table 1. *Protein content of the homogenates*

All tissues were homogenized with one part (v/w) of 0·9% NaCl soln.

Source of homogenate	Protein (mg./ml.)
Stomach	11·5
Duodenum	56·0
Upper jejunum	63·0
Lower jejunum	41·0
Upper ileum	30·0
Lower ileum	34·0
Colon	25·0
Pancreas	45·0

Invertase activity was low in the duodenum (7·5 units/ml. of homogenate) and was greatest in the lower jejunum and the ileum (50–70 units/ml.) (Fig. 1). Even the homogenate prepared from a section of the lower ileum, cut just proximal to the ileocoecal valve, had strong invertase activity (54 units/ml.).

Maltase activity of the homogenate from the duodenum was 66 units/ml., which is nearly 10 times its invertase activity. The maltase activity also increased in the lower part of the small intestine (Fig. 1) but not to the same extent as the invertase activity. In the homogenates from the lower jejunum and the ileum the maltase activity was 100–150 units/ml., which is two to three times the invertase activity.

The maltase activity of the small intestine of the pig has recently been demonstrated to be effected by a mixture of three different maltases (maltase I–III). The results of the determination of each of these enzymes, by the methods described earlier (Dahlqvist, 1959, 1960*c*, *d*), with 28 mM-substrate

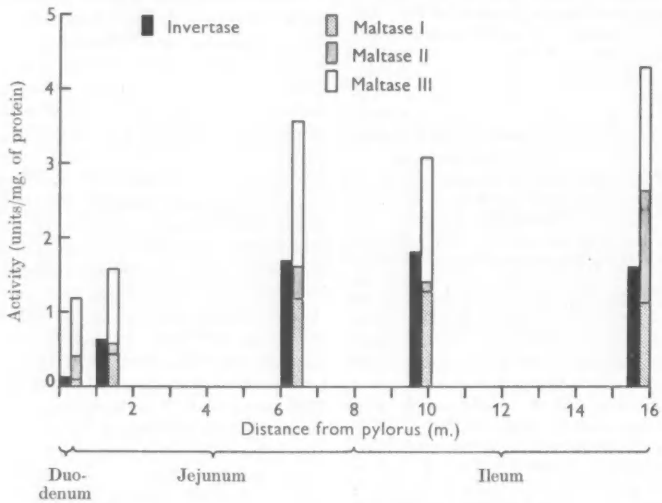

Fig. 1. Invertase and maltase activities of homogenates of mucosa from different parts of the small intestine of an adult pig. The three different maltases (maltase I–III) present in such preparations have been measured separately by the methods described earlier (Dahlqvist, 1959, 1960*c*, *d*).

concentration (Dahlqvist, 1960*d*), are seen in Fig. 1. Maltase II activity, as compared with maltase III, is unusually low. Other experiments with homogenates prepared from the small intestine of the pig have demonstrated that maltase II activity is usually equal to or somewhat greater than maltase III activity; maltase II and maltase III together usually exert 80–85 % of the total activity (Dahlqvist, 1960*d*).

Isomaltase activity, which is to some extent due to the presence of maltase II and maltase III but mostly (75 %) to a 'specific' isomaltase (Dahlqvist, 1960*b*), had a distribution in the small intestine which was similar to that of the invertase and maltase activities (Fig. 2), i.e. the isomaltase activity per ml. of homogenate rose from a low value (1·5 units/ml.) in the preparation from the duodenum to about 30 units/ml. in the homogenates from the lower jejunum and the ileum.

Trehalase activity, which is due to a specific enzyme (Dahlqvist, 1960*a*), showed a distribution which was quite opposite to that of the enzymes described above (Fig. 3). The trehalase activity was greatest in the preparations from the duodenum and the jejunum (15–20 units/ml. of homogenate) but decreased in the lower part of the small intestine.

Lactase activity was greater in the homogenate from the upper jejunum (18 units/ml.) than in that from the duodenum (9 units/ml.), but was practically absent from the homogenates of the ileum (Fig. 4). The cellobiase activity had the same distribution as the lactase activity, and the ratio of cellobiase to lactase was the same in homogenates from all parts of the small intestine (Fig. 4). This suggests the possibility that the cellobiase and lactase activities are caused by one and the same enzyme. The relation between these two activities is under investigation in our laboratory.

Amylase activity was present in all the homogenates of small-intestinal mucosa, but was low (200–700 units/ml.) when compared with the activity in the homogenate of the pancreas (see below). It is quite possible that at least some of the activity of the mucosa homogenate was caused by contamination with intestinal contents, which contain considerable amounts of pancreatic amylase.

The amylase activity of the homogenates, prepared from different parts along the small intestine, varied irregularly.

Dextranase activity was low in all the homogenates, but increased in a distal direction in the small intestine from 1 unit/ml. in the homogenate from the duodenum to 5·5 units/ml. in that from the lower ileum (Fig. 5). Since the homogenate of the pancreas had no dextranase activity (see below) the activity of the homogenates of intestinal mucosa could not be caused by contamination with pancreatic juice. The low dextranase activity of the samples, together with the localization of this activity to the distal part of the small intestine, might suggest that the dextranase activity was

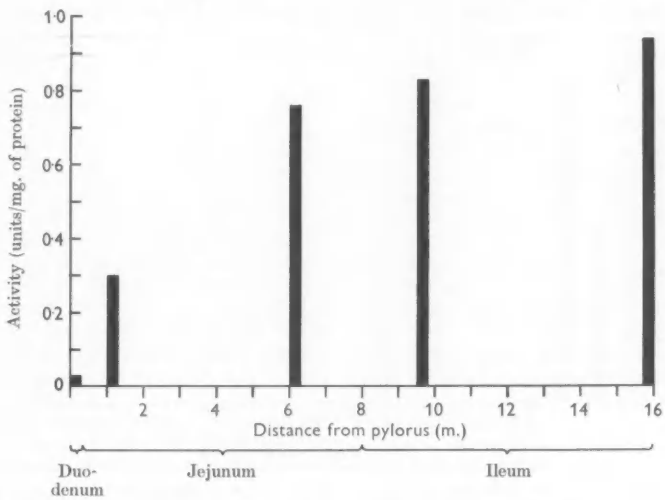

Fig. 2. Total isomaltase activity of homogenates of mucosa from different parts of the small intestine of an adult pig. The isomaltase activity of such preparations is exerted by a mixture of three different enzymes as described previously (Dahlqvist, 1960*b*).

caused by contamination with intestinal bacteria. Against this possibility is, however, the low dextranase activity of the homogenate of mucosa from the colon (Table 2).

Colon. The mucosa from the colon was scraped off a few centimetres distal to the ileocoecal valve. The carbohydrase activities of the homogenate are seen in Table 2. Although most of the carbohydrase activities were present in this preparation, too, they were present only in small amounts, as compared with the activities of the homogenates of small intestinal mucosa.

Pancreas. The homogenate of the pancreas was kept chilled with crushed ice until just before assay, and all assays were completed within a few hours after the homogenization.

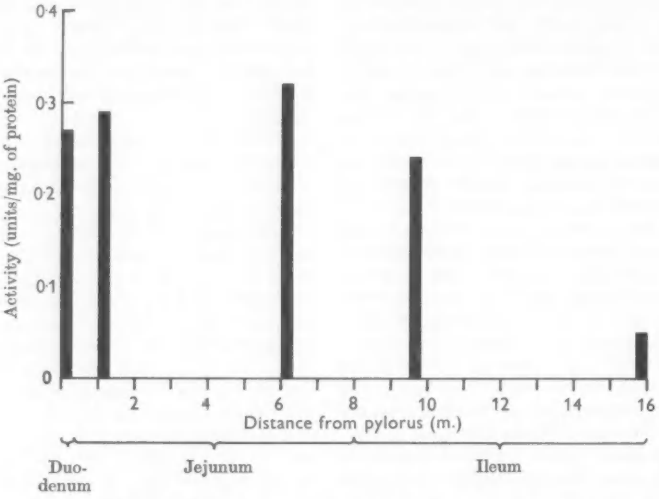

Fig. 3. Trehalase activity of homogenates of mucosa from different parts of the small intestine of an adult pig.

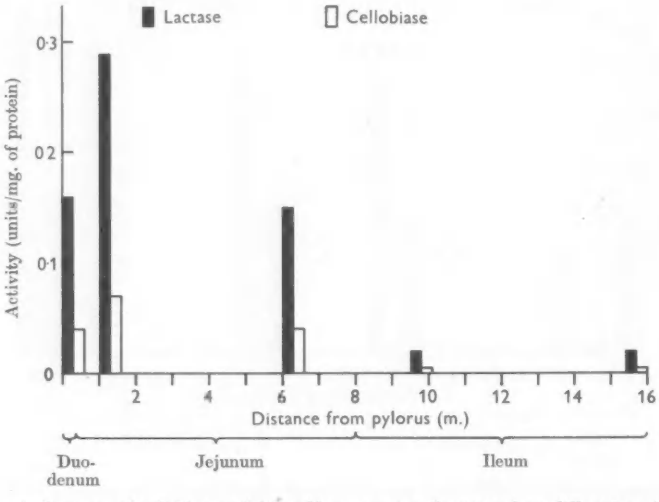

Fig. 4. Lactase and cellobiase activities of homogenates of mucosa from different parts of the small intestine of an adult pig.

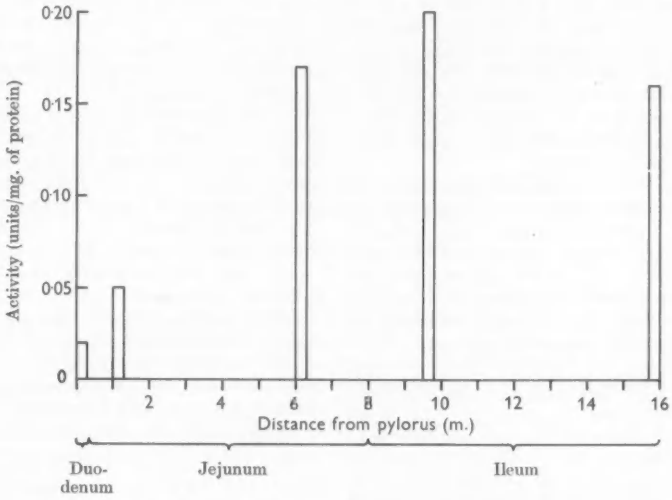

Fig. 5. Dextranase activity of homogenates of mucosa from different parts of the small intestine of an adult pig.

Table 2. *Carbohydrase activities of the homogenate of mucosa from the colon of an adult pig*

Protein content of the homogenate was 25 mg./ml.

Enzyme	Activity (units/ml. of homogenate)
Invertase	8
Maltase I	6
Maltase II	
Maltase III	
Isomaltase	2
Trehalase	0·5
Lactase	0
Cellobiase	0
Amylase	250
Dextranase	1

Only two carbohydrase activities were recognized, namely very active amylase (80 000 units/ml.) and weak maltase (12 units/ml.) activity. This amylase activity was more than 100 times that of the homogenate of small-intestinal mucosa, which suggests that it is mainly pancreatic amylase which effects the hydrolysis of starch in the small intestine.

The pancreatic-maltase activity, by contrast, was very weak, whether compared with that of the preparations of small-intestinal mucosa (see above) or with the pancreatic-amylase activity. It may therefore be concluded that the pancreatic maltase is without physiological importance for the hydrolysis of maltose in the small intestine.

DISCUSSION

It is apparent that the disaccharidases are mainly localized in the small intestinal mucosa. The weak maltase activity of the pancreas and the weak disaccharidase activities of the mucosa of the colon cannot contribute to any considerable extent to the hydrolysis of ingested disaccharides.

The findings about the location of the disaccharidase activities along the small intestine seem remarkable. From the present investigation it appears that there exist two major groups of pig-intestinal disaccharidase activities, according to their distribution along the small intestine. One group (invertase, maltase and isomaltase) is localized mainly in the distal part of the small intestine, and the other group (trehalase, lactase and cellobiase) is localized mainly in the proximal part of the small intestine. It seems tempting to conclude that the corresponding disaccharides are hydrolysed and absorbed in the corresponding parts of the small intestine. Our previous finding that lactose is absorbed in the proximal part of the small intestine of humans (Borgström *et al.* 1957) agrees with this idea, but further experimental studies are required.

The location of the invertase activity differs from the findings by some earlier authors who have studied other species. Euler & Svanberg (1921) analysed a human small intestine and found that the invertase activity (per cm.^{2} of intestine) was highest in the upper part of the jejunum, whereas

the lower ileum had only 5% of this activity. Recently Ammon & Henning (1956) have found a similar distribution of invertase in rabbits. In the present paper figures are reported for one pig only. Experiments with pieces of intestine of several other pigs have, however, demonstrated that there are hardly any individual variations in the location of the different carbohydrases.

The location of the lactase activity along the small intestine agrees well with that given in earlier reports. Cajori (1935) has found that the lactase activity of mucosa preparations from dog small intestine was 30% greater in preparations from the jejunum than in those from the duodenum (calculated per gram of mucosa), and Heilskov (1951) has found that the lactase activity of the mucosa in different mammals (rabbits, cows and human foetuses) diminishes in the distal segments of the small intestine.

That the homogenate of the pancreas contained a powerful amylase but no disaccharidases, except a very weak maltase, is in accordance with earlier reports, which state that the pancreas contains amylase and maltase, but no invertase (Brown & Heron, 1880; Oppenheimer, 1925), lactase (Heilskov, 1951) or trehalase (Frèrejacque, 1953). The absence of disaccharidase activities from the pancreatic juice can also be deduced from the finding that the small-intestinal contents contain amylase but essentially no disaccharidases (Ammon & Henning, 1956; Borgström *et al.* 1957).

The fact that the homogenate of the pancreas had no dextranase activity demonstrates that intestinal dextranase is a specific enzyme, distinguished from amylase. Like the disaccharidases the dextranase is located in the intestinal mucosa.

SUMMARY

1. The carbohydrase activities of homogenates prepared from mucosa of the stomach, small intestine and colon, and from pancreatic tissue, of an adult pig have been studied.

2. The preparation from the stomach did not contain any carbohydrases except a very weak amylase activity, which may have been caused by contamination with saliva from the gastric contents.

3. The preparations from the small intestine had powerful disaccharidase activities which showed different locations along the small intestine: one group of activities (invertase, maltase and isomaltase) was mainly localized in the distal part of the small intestine, and another group (trehalase, lactase and cellobiase) was localized in the proximal part. This may indicate that different disaccharides are absorbed in different parts of the small intestine.

4. The preparation from the small intestine also had amylase activity, which, however, was low compared with that of the pancreas homogenate and may have been due to contamination with pancreatic juice.

5. Dextranase activity was present in the preparations from the small intestine, but not in those from the pancreas. The dextranase activity is thus caused by a specific enzyme which has its origin in the small intestinal mucosa.

6. The preparation from the colon also had carbohydrase activities, although these activities were weak compared with those of the small intestinal mucosa.

7. The preparation from the pancreas had very powerful amylase activity, and the pancreas seems to be the main source of the amylase of the intestinal contents. This preparation also had weak maltase activity. The preparation from the pancreas had no other disaccharidase activities.

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Thermodynamic Quantities for the Dissociation Equilibria of Biologically Important Compounds

8. THE FIRST AND SECOND ACID DISSOCIATIONS OF ARGININE*

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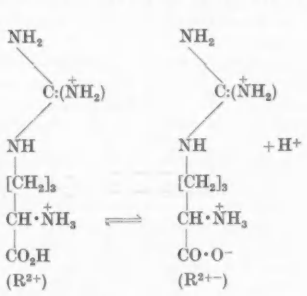
Although the thermodynamics of the acid dissociations of a number of amino acids have been investigated (see, for example, Edsall & Wyman, 1958) no detailed study of arginine appears to have been made. Consequently we have determined the values of pK_{1a} (carboxyl) and pK_{2a} (ammonium) of arginine, on the molal scale, by e.m.f. measurements of cells without liquid junction from 0° to 55° at 5° intervals.

THEORY

Values of pK_{1a}

This dissociation constant refers to the equilibrium:



for the study of which the cell:

$$\text{Pd, } H_2(1 \text{ atm.}) \mid \text{arginine HCl } (m_1), \text{ HCl } (m_2) \mid \text{AgCl, Ag} \quad (1)$$

was used. In these solutions

$$m_{R^{2+}} = m_2 - m_{H^+}, \quad m_{R^{2+-}} = m_1 - m_2 + m_{H^+},$$
$$m_{Cl^-} = m_1 + m_2 \quad \text{and} \quad I = m_1 + 2m_2 - m_{H^+}.$$

From the equations for the e.m.f. of the cell and for the first dissociation of arginine, we get

$$pK_{1a} = \text{pwH} + \log (m_{R^{2+}}/m_{R^{2+-}}) + \log (\gamma_{R^{2+}} \cdot \gamma_{Cl^-}/\gamma_{R^{2+-}}) \quad (2)$$

where:

$$\text{pwH} = \boldsymbol{F}(E - E_0)/(\boldsymbol{RT} \ln 10) + \log m_{Cl^-}.$$

The individual activity coefficients in the last term of eqn. (2) may each be represented, up to $I = 0{\cdot}1$, by the equation

$$-\log \gamma_i = z_i^2 \boldsymbol{A} I^{\frac{1}{2}} + B_i I + C_i I^{\frac{3}{2}} \quad (3)$$

where z_i is the charge on the ith ion, $\boldsymbol{A}$ is the Debye–Hückel constant and B_i and C_i are arbitrary constants (Datta & Grzybowski, 1958*a*).

Substituting for $m_{R^{2+}}$ and $m_{R^{2+-}}$, we may set

$$Y_1 = \text{pwH} + \log [(m_2 - m_{H^+})/(m_1 - m_2 + m_{H^+})] \quad (4)$$

then, combining eqns. (2), (3) and (4), we obtain

$$pK_{1a} = Y_1 - 4\boldsymbol{A} I^{\frac{1}{2}} - B_1 I - C_1 I^{\frac{3}{2}}. \quad (5)$$

The values of pK_{1a}, B_1 and C_1 were determined by fitting values of $Y_1' = (Y_1 - 4\boldsymbol{A} I^{\frac{1}{2}})$ to the equation $Y_1' = pK_{1a} + B_1 I + C_1 I^{\frac{3}{2}}$, by the method of least squares.

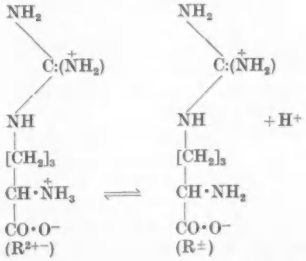
In eqn. (4), and for the evaluation of I, m_{H^+} was calculated from the relation

$$m_{H^+} = \text{antilog } [-(\text{pwH} + 2 \log \gamma_{\pm HCl})].$$

The values of $\log \gamma_{\pm HCl}$ being found from an equation, of the form of eqn. (3), fitted the data of Bates & Bower (1954). An approximate value of I was used to find a preliminary value of m_{H^+}, I was then corrected and final values of I and m_{H^+} were found by iteration. Although the use of $\log \gamma_{\pm HCl}$ is to some extent arbitrary, since the medium effect of arginine is neglected, it is nevertheless one of the most reasonable approximations available. Any error introduced by it should largely disappear on extrapolation to infinite dilution.

Values of pK_{2a}

This dissociation constant refers to the equilibrium:



* Part 7: Datta & Grzybowski (1958*c*).

for the study of which the cell:

$$\text{Pd, } H_2 \text{ (1 atm.)} \mid \text{arginine HCl } (m_1), \quad \text{KOH } (m_2), \text{ KCl } (m_3) \mid \text{AgCl, Ag} \qquad (6)$$

was used.

In these solutions 'complexing' of Ag^+ ions by the species $R^{\pm}$ occurs; this increases the solubility of the AgCl of the Ag, AgCl electrode, and consequently corrections, Δm_{Cl^-} and $\Delta m_{R^{\pm}}$, have to be applied to m_{Cl^-} and $m_{R^{\pm}}$. These corrections are given by

$$\Delta m_{Cl^-} = \frac{K_s}{K_{1l}} \frac{m_{R^{\pm}}}{m_{Cl^-}} \left[1 + \frac{m_{R^{\pm}}}{K_{2l}}\right]$$

and

$$\Delta m_{R^{\pm}} = \frac{K_s}{K_{1l}} \frac{m_{R^{\pm}}}{m_{Cl^-}} \left[1 + \frac{2m_{R^{\pm}}}{K_{2l}}\right]$$

(Datta & Grzybowski, 1958*a*), where K_s is the solubility product of AgCl calculated from eqn. (10) of Owen & Brinkley (1938) and K_{1l} and K_{2l} are the first and second instability constants of the silver arginine complexes (Datta & Grzybowski, 1959).

Consequently we have the following relations:

$$m_{R^{\pm}-} = m_1 - m_2 + m_{OH^-},$$

$$m_{R^{\pm}} = m_2 - m_{OH^-} - \Delta m_{R^{\pm}},$$

$$m_{Cl^-} = m_1 + m_3 + \Delta m_{Cl^-}$$

and

$$I = m_1 + m_3 + \Delta m_{Cl^-} + m_{OH^-}.$$

The hydroxide-ion concentration was calculated from: m_{OH^-} = antilog $[\text{pwH} - \text{p}K_w]$, where $\text{p}K_w$ is the negative logarithm of the ion product of water, calculated from the equation given by Robinson & Stokes (1955).

From the equations for the e.m.f. of the cell and for the second dissociation of arginine, we get

$$\text{p}K_{2a} = \text{pwH} + \log (m_{R^{\pm}-}/m_{R^{\pm}}) + \log (\gamma_{R^{\pm}-} \cdot \gamma_{Cl^-}/\gamma_{R^{\pm}}). \qquad (7)$$

Substituting for $m_{R^{\pm}-}$ and $m_{R^{\pm}}$ we may set

$$Y_2 = \text{pwH} + \log [(m_1 - m_2 + m_{OH^-})/(m_2 - m_{OH^-} - \Delta m_{R^{\pm}})]. \qquad (8)$$

Then combining eqns. (7) and (8) and representing the activity coefficients by eqn. (3) again, we have

$$\text{p}K_{2a} = Y_2 - 2AI^{\frac{1}{2}} - B_2 I - C_2 I^{\frac{3}{2}}. \qquad (9)$$

The values of B_2 and C_2 and $\text{p}K_{2a}$ were determined, as for $\text{p}K_{1a}$, by fitting values of $Y_2' = (Y_2 - 2AI^{\frac{1}{2}})$ to the equation $Y_2' = \text{p}K_{2a} + B_2 I + C_2 I^{\frac{3}{2}}$, by the method of least squares.

The method of extrapolation to $I = 0$ used here (eqns. 5 and 9) was selected because it had previously been used successfully for $\text{p}K_{2a}$ of glycine (Datta & Grzybowski, 1958*a*) and also because the constants A, B and C can be used to compute the 'apparent' pK values at given ionic strengths.

METHODS

The apparatus and technique for the measurements in cells without liquid junction were, in general, the same as have been described previously (Ashby, Crook & Datta, 1954; Ashby, Clarke, Crook & Datta, 1955). Stable e.m.f. values were not obtained with alkaline arginine solutions when the hydrogen electrodes consisted of Pt foil coated with Pt black. When, however, the Pt foil was coated with Pd black, by electrolysis, the e.m.f. values obtained were both stable and reproducible. Consequently in all the experiments with arginine the Pt foil of the hydrogen electrodes was palladized by electrolysis in a solution of 3 g. of $PdCl_2,2H_2O$ in 100 ml. of 0·4 N-HCl, with a Pd anode, at approx. 0·2 A/cm.^{2} for about 5 min. Electrolysis was continued until the plates were evenly covered with a thin black coating of Pd.

L-Arginine hydrochloride was purified by conversion into benzylidenearginine and subsequent hydrolysis with bromine-free HCl [*Org. Synth.* (1943), *Coll. Vol.* **2**, 49]. The arginine hydrochloride was recrystallized and dried *in vacuo* over P_2O_5. All solutions used in the cells without liquid junction were collected and the arginine was recovered as benzylidenearginine. This material was converted into arginine hydrochloride and used in subsequent sets of experiments.

The purity of the arginine hydrochloride was estimated by gravimetric chloride determination, and in some instances also by potentiometric titration. In all specimens the purity was found to be between 99·5 and 100%; the impurity was considered to be water, the necessary corrections being made to the weights.

Constant-boiling HCl was prepared from AnalaR-grade concentrated acid, its concentration being determined from the mean atmospheric pressure at the time of distillation and checked by gravimetric chloride determinations as AgCl.

Carbonate-free KOH was prepared electrolytically from either AnalaR KCl or AnalaR KOH in a laboratory-scale Castner–Kellner cell. Its concentration was determined by titration against the constant-boiling HCl.

Potassium chloride (AnalaR) was freed from bromine by chlorination of a concentrated solution. It was reprecipitated by HCl gas, dried, ignited at 350° and finally fused in an atmosphere of dry N_2.

The values of E_0 for each of the Ag–AgCl electrodes used were determined by measurements of the e.m.f. of cells of the type:

$$\text{Pd, } H_2 \text{ (1 atm.)} \mid \text{HCl } (m) \mid \text{AgCl, Ag} \qquad (10)$$

at 25°. The HCl solutions were approx. 0·05 molal and their activity coefficients were calculated from the parameters of eqn. (3) fitted to the values given in Table 3 of Bates & Bower (1954). The E_0 values at other temperatures were calculated by using Bates & Bower's temperature coefficient.

RESULTS

The values of $(E - E_0)$ observed, the molalities of the solutions used and of the extrapolation functions Y_1 and Y_2 calculated for $\text{p}K_{1a}$ and $\text{p}K_{2a}$ respectively are given in Tables 1 and 2. The values of the constants B and C of eqns. (5) and (9) and the

Table 1. *First dissociation of arginine* ($\text{p}K_{1a}$): *variations of molalities* (m) *and ionic strengths* (I) *of solutions, e.m.f. values of cell* 1 ($E - E_0$) *and extrapolation functions* (Y_1)

Table 1. *First dissociation of arginine* (pK_{1a}): *variations of molalities* (m) *and ionic strengths* (I) *of solutions, e.m.f. values of cell* 1 ($E - E_0$) *and extrapolation functions* (Y_1)

m_1 = molality of arginine hydrochloride; m_2 = molality of HCl; $I = m_1 + 2m_2 - m_{H^+}$; $(E - E_0)$ is given in absolute volts corrected to 1 atm. hydrogen pressure and rounded temperatures. The values of E_0 used were determined for each Ag–AgCl electrode by measurements in cell 10; $Y_1 = pwH + \log(m_{R^{2+}}/m_{R^{2+}-}$).

Set 1

	$10^2 m_1$	0·5238	0·8892	1·303	1·647	2·539	2·985	3·365	3·604	3·616	3·878	4·406
	$10^2 m_2$	0·2003	0·3401	0·4982	0·6299	0·9712	1·142	1·287	1·378	1·383	1·483	1·685
Temp.												
0°	$10^5 (E - E_0)$	27 504	25 564	24 268	23 524	22 260	21 824	21 503	21 330	21 332	21 145	20 833
	$10^2 I$	0·7862	1·375	2·060	2·641	4·171	4·944	5·604	6·022	6·046	6·501	7·426
	Y_1	2·0633	2·0986	2·1285	2·1504	2·1989	2·2192	2·2320	2·2413	2·2446	2·2505	2·2670
10°	$10^5 (E - E_0)$	28 452	26 420	25 064	24 282	22 957	22 495	22 159	21 972	21 975	21 781	21 449
	$10^2 I$	0·7825	1·368	2·050	2·628	4·154	4·925	5·584	6·000	6·024	6·479	7·402
	Y_1	2·0224	2·0589	2·0903	2·1125	2·1628	2·1822	2·1956	2·2038	2·2074	2·2138	2·2297
20°	$10^5 (E - E_0)$	29 416	27 302	25 887	25 071	23 687	23 206	22 855	22 662	22 663	22 462	22 115
	$10^2 I$	0·7798	1·363	2·043	2·619	4·142	4·911	5·569	5·985	6·009	6·463	7·385
	Y_1	1·9925	2·0319	2·0638	2·0865	2·1375	2·1576	2·1710	2·1798	2·1831	2·1900	2·2059
30°	$10^5 (E - E_0)$	30 396	28 201	26 731	25 886	24 447	23 946	23 582	23 380	23 374	23 169	22 811
	$10^2 I$	0·7780	1·359	2·037	2·613	4·132	4·901	5·558	5·973	5·996	6·450	7·371
	Y_1	1·9724	2·0126	2·0454	2·0696	2·1211	2·1413	2·1550	2·1635	2·1652	2·1730	2·1898
40°	$10^5 (E - E_0)$	31 385	29 115	27 593	26 727	25 231	24 714	24 336	24 126	24 127	23 910	23 540
	$10^2 I$	0·7770	1·358	2·035	2·611	4·129	4·897	5·554	5·969	5·992	6·446	7·367
	Y_1	1·9612	2·0039	2·0368	2·0645	2·1141	2·1350	2·1486	2·1568	2·1602	2·1670	2·1840
50°	$10^5 (E - E_0)$	32 381	30 041	28 472	27 577	26 035	25 504	25 119	24 902	24 897	24 673	24 298
	$10^2 I$	0·7761	1·357	2·033	2·608	4·126	4·893	5·550	5·965	5·988	6·441	7·363
	Y_1	1·9520	1·9979	2·0326	2·0598	2·1108	2·1322	2·1472	2·1555	2·1575	2·1642	2·1829

Set 2

	$10^2 m_1$	0·9341	1·293	1·581	1·935	2·362	2·741	3·114	3·340	3·555	3·999	4·421
	$10^2 m_2$	0·2370	0·3282	0·4012	0·4910	0·5993	0·6954	0·7900	0·8475	0·9020	1·015	1·122
5°	$10^5 (E - E_0)$	27 056	25 950	25 313	24 698	24 126	23 714	23 385	23 198	23 042	22 761	22 504
	$10^2 I$	1·276	1·793	2·213	2·733	3·365	3·928	4·485	4·823	5·144	5·811	6·443
	Y_1	2·0712	2·0964	2·1159	2·1349	2·1568	2·1722	2·1892	2·1960	2·2036	2·2204	2·2290
15°	$10^5 (E - E_0)$	27 959	26 799	26 135	25 491	24 892	24 457	24 111	23 917	23 754	23 456	23 190
	$10^2 I$	1·271	1·787	2·206	2·725	3·356	3·918	4·474	4·812	5·133	5·799	6·430
	Y_1	2·0393	2·0644	2·0854	2·1050	2·1270	2·1422	2·1590	2·1664	2·1743	2·1905	2·1996
25°	$10^5 (E - E_0)$	28 882	27 676	26 984	26 314	25 688	25 236	24 873	24 672	24 502	24 191	23 916
	$10^2 I$	1·268	1·783	2·201	2·719	3·349	3·910	4·465	4·803	5·124	5·790	6·420
	Y_1	2·0160	2·0424	2·0642	2·0844	2·1064	2·1221	2·1387	2·1462	2·1544	2·1707	2·1804
35°	$10^5 (E - E_0)$	29 817	28 570	27 854	27 158	26 506	26 037	25 666	25 456	25 281	24 958	24 673
	$10^2 I$	1·266	1·780	2·198	2·716	3·345	3·905	4·461	4·798	5·119	5·784	6·415
	Y_1	1·9999	2·0285	2·0514	2·0720	2·0935	2·1094	2·1275	2·1350	2·1434	2·1598	2·1697
45°	$10^5 (E - E_0)$	30 767	29 480	28 743	28 028	27 354	26 865	26 486	26 268	26 087	25 756	25 463
	$10^2 I$	1·265	1·779	2·196	2·714	3·342	3·902	4·458	4·795	5·116	5·781	6·412
	Y_1	1·9895	2·0203	2·0447	2·0667	2·0884	2·1034	2·1229	2·1300	2·1387	2·1558	2·1661
55°	$10^5 (E - E_0)$	31 733	30 399	29 651	28 918	28 221	27 715	27 334	27 100	26 915	26 573	26 274
	$10^2 I$	1·264	1·777	2·196	2·713	3·341	3·901	4·458	4·794	5·115	5·780	6·410
	Y_1	1·9858	2·0148	2·0437	2·0670	2·0884	2·1030	2·1248	2·1299	2·1390	2·1559	2·1668

Table 2. *Second dissociation of arginine* (pK_{2a}): *variations of molalities* (m) *and ionic strengths* (I) *of solutions, e.m.f. values of cell* 6 ($E - E_0$) *and extrapolation functions* (Y_2)

m_1 = molality of arginine hydrochloride; m_2 = molality of KOH; m_3 = molality of KCl; $I = m_1 + m_2 + \Delta m_{Cl^-} + m_{OH^-}$; $(E - E_0)$ is given in absolute volts corrected to 1 atm. hydrogen pressure and rounded temperatures; the values of E_0 used were determined for each Ag–AgCl electrode by measurements in cell 10; $Y_2 = pwH + \log (m_{R^{2+}}/m_{R^+})$.

Set 1

	$10^2 m_1$	0·5180	0·5948	0·8189	0·9076	1·280	1·383	1·701	1·797	2·032	2·064
	$10^2 m_2$	0·2554	0·2954	0·4066	0·4475	0·6310	0·6869	0·8446	0·8860	1·009	1·018
	$10^2 m_3$	0·3542	0·3703	0·5483	0·6206	0·8751	0·9391	1·159	1·229	1·329	1·411
Temp.											
0°	$10^5(E - E_0)$	64 242	64 057	63 327	63 073	62 399	62 264	61 862	61 721	61 534	61 461
	$10^2 I$	0·8730	0·9660	1·368	1·529	2·156	2·324	2·861	3·027	3·362	3·476
	Y_2	9·8090	9·8127	9·8288	9·8360	9·8606	9·8622	9·8781	9·8826	9·8875	9·8946
10°	$10^5(E - E_0)$	64 853	64 660	63 906	63 645	62 944	62 801	62 386	62 243	62 051	61 974
	$10^2 I$	0·8731	0·9662	1·368	1·529	2·156	2·324	2·861	3·027	3·362	3·477
	Y_2	9·4998	9·5032	9·5195	9·5271	9·5512	0·5522	9·5685	9·5735	9·5789	9·5856
20°	$10^5(E - E_0)$	65 485	65 292	64 515	64 238	63 512	63 371	62 938	62 791	62 596	62 510
	$10^2 I$	0·8733	0·9664	1·369	1·529	2·156	2·324	2·861	3·027	3·362	3·477
	Y_2	9·2154	9·2199	9·2367	9·2433	9·2671	9·2692	9·2850	9·2900	9·2961	9·3017
30°	$10^5(E - E_0)$	66 134	65 940	65 136	64 850	64 101	63 957	63 509	63 351	63 155	63 066
	$10^2 I$	0·8735	0·9666	1·369	1·530	2·157	2·324	2·861	3·027	3·363	3·477
	Y_2	8·9526	8·9578	8·9745	8·9810	9·0050	9·0075	9·0232	9·0273	9·0342	9·0398
40°	$10^5(E - E_0)$	66 800	66 602	65 774	65 477	64 709	64 559	64 096	63 941	63 734	63 640
	$10^2 I$	0·8737	0·9667	1·369	1·530	2·157	2·324	2·862	3·028	3·363	3·477
	Y_2	8·7094	8·7149	8·7319	8·7381	8·7630	8·7652	8·7807	8·7862	8·7924	8·7978
50°	$10^5(E - E_0)$	67 475	67 275	66 424	66 112	65 333	65 179	64 691	64 544	64 326	64 227
	$10^2 I$	0·8738	0·9669	1·369	1·530	2·157	2·325	2·862	3·028	3·363	3·478
	Y_2	8·4826	8·4890	8·5062	8·5116	8·5382	8·5408	8·5548	8·5622	8·5676	8·5726
	$10^2 m_1$	2·388	2·377	2·594	2·886	3·105	3·592	3·868	4·388	4·880	
	$10^2 m_2$	1·186	1·172	1·288	1·423	1·531	1·771	1·908	2·164	2·406	
	$10^2 m_3$	1·562	1·625	1·679	1·974	2·123	2·456	2·645	3·000	3·337	
Temp.											
0°	$10^5(E - E_0)$	61 232	61 171	61 069	60 810	60 655	60 379	60 252	60 008	59 814	
	$10^2 I$	3·951	4·003	4·274	4·861	5·229	6·049	6·515	7·390	8·218	
	Y_2	9·9020	9·9024	9·9061	9·9200	9·9231	9·9354	9·9442	9·9538	9·9641	
10°	$10^5(E - E_0)$	61 737	61 670	61 566	61 297	61 137	60 850	60 716	60 460	60 261	
	$10^2 I$	3·951	4·003	4·275	4·862	5·229	6·049	6·516	7·391	8·219	
	Y_2	9·5929	9·5928	9·5967	9·6106	9·6137	9·6258	9·6343	9·6434	9·6539	
20°	$10^5(E - E_0)$	62 270	62 199	62 092	61 812	61 647	61 351	61 212	60 950	60 743	
	$10^2 I$	3·952	4·003	4·275	4·862	5·229	6·050	6·516	7·391	8·219	
	Y_2	9·3100	9·3095	9·3135	9·3272	9·3304	9·3427	9·3510	9·3606	9·3712	
30°	$10^5(E - E_0)$	62 819	62 745	62 634	62 346	62 176	61 870	61 725	61 456	61 242	
	$10^2 I$	3·952	4·004	4·275	4·862	5·230	6·050	6·516	7·391	8·219	
	Y_2	9·0484	9·0477	9·0517	9·0655	0·0689	9·0811	9·0892	9·0992	9·1098	
40°	$10^5(E - E_0)$	63 390	63 315	63 199	62 902	62 725	62 402	62 258	61 982	61 763	
	$10^2 I$	3·952	4·004	4·275	4·862	5·230	6·050	6·516	7·391	8·219	
	Y_2	8·8069	8·8064	8·8102	8·8243	8·8273	8·8386	8·8476	8·8578	8·8685	
50°	$10^5(E - E_0)$	63 971	63 899	63 776	63 473	63 295	62 952	62 809	62 527	62 291	
	$10^2 I$	3·952	4·004	4·276	4·862	5·230	6·050	6·517	7·392	8·220	
	Y_2	8·5822	8·5826	8·5858	8·6003	8·6042	8·6139	8·6236	8·6343	8·6436	

Set 2 ($m_2 = 0$)

	$10^2 m_1$	0·9503	1·887	3·011	4·060	4·445	5·775	7·046	8·203
	$10^2 m_3$	0·3152	0·6258	0·9987	1·346	1·474	1·915	2·337	2·721
5°	$10^5(E - E_0)$	62 762	61 342	60 401	59 778	59 617	59 089	58 732	58 412
	$10^2 I$	0·9508	1·888	3·012	4·061	4·447	5·776	7·048	8·205
	Y_2	9·6560	9·6962	9·7284	9·7452	9·7554	9·7734	9·7950	9·8030
15°	$10^5(E - E_0)$	63 336	61 862	60 887	60 250	60 083	59 543	59 161	58 842
	$10^2 I$	0·9509	1·888	3·012	4·061	4·447	5·776	7·048	8·205
	Y_2	9·3619	9·4015	9·4338	9·4520	9·4621	9·4813	9·5009	9·5110
25°	$10^5(E - E_0)$	63 924	62 399	61 396	60 740	60 567	60 014	59 613	59 294
	$10^2 I$	0·9510	1·888	3·012	4·061	4·447	5·777	7·048	8·205
	Y_2	9·0898	9·1295	9·1626	9·1815	9·1915	9·2116	9·2303	9·2423
35°	$10^5(E - E_0)$	64 522	62 947	61 918	61 240	61 066	60 496	60 079	59 757
	$10^2 I$	0·9511	1·888	3·012	4·061	4·447	5·777	7·048	8·205
	Y_2	8·8374	8·8770	8·9114	8·9302	8·9411	8·9615	8·9796	8·9929
45°	$10^5(E - E_0)$	65 139	63 502	62 455	61 741	61 579	60 985	60 560	60 230
	$10^2 I$	0·9512	1·888	3·012	4·061	4·447	5·777	7·049	8·205
	Y_2	8·6038	8·6415	8·6783	8·6949	8·7086	8·7281	8·7471	8·7608
55°	$10^5(E - E_0)$	65 760	64 080	62 998	62 290	62 104	61 490	61 052	60 717
	$10^2 I$	0·9513	1·888	3·013	4·061	4·447	5·777	7·049	8·205
	Y_2	8·3850	8·4241	8·4606	8·4813	8·4922	8·5115	8·5306	8·5450

Table 3. *First dissociation of arginine: values used for the constants B_1 and C_1 of eqn. (5) and the values of pK_{1a}*

pK_{1a} (obs.) was obtained from eqn. (5) and pK_{1a} (calc.) from eqn. (11). pK_{1a} (calc.) $= \frac{1087{\cdot}6}{T} - 4{\cdot}7526 + 0{\cdot}0098197T$; T (min.) $= 59{\cdot}7°$; pK_{1a} (min.) $= 1{\cdot}783$. σ is the standard error (Please, 1954).

Set	Temp.	B_1	C_1	pK_{1a} (obs.)	pK_{1a} (calc.)
1	0°	+3·851	−5·383	1·9140	1·911
2	5	+3·349	−3·814	1·8847	1·889
1	10	+3·635	−4·533	1·8697	1·869
2	15	+3·245	−3·311	1·8488	1·851
1	20	+3·522	−4·102	1·8374	1·836
2	25	+3·143	−2·851	1·8217	1·823
1	30	+3·408	−3·560	1·8138	1·812
2	35	+2·985	−2·266	1·8013	1·803
	37	—	—	—	1·799
1	40	+3·249	−2·879	1·7995	1·795
2	45	+2·690	−1·242	1·7852	1·790
1	50	+3·100	−2·325	1·7871	1·786
2	55	+2·276	+0·274	1·7731	$\sigma = 0{\cdot}003$

Table 4. *Second dissociation of arginine: values used for the constants B_2 and C_2 of eqn. (9) and the values of pK_{2a}*

pK_{2a} (obs.) was obtained from eqn. (9) and pK_{2a} (calc.) from eqn. (11). pK_{2a} (calc.) $= \frac{2643{\cdot}6}{T} - 0{\cdot}8783 + 0{\cdot}003363T$. σ is the standard error (Please, 1954).

Set	Temp.	B_2	C_2	pK_{2a} (obs.)	pK_{2a} (calc.)
1	0°	−0·115	+1·876	9·7178	9·718
2	5	−0·110	+2·122	9·5626	9·561
1	10	−0·080	+1·935	9·4073	9·410
2	15	−0·052	+1·974	9·2669	9·265
1	20	−0·001	+1·778	9·1226	9·125
2	25	−0·012	+1·876	8·9936	8·991
1	30	+0·057	+1·691	8·8589	8·861
2	35	−0·004	+1·848	8·7390	8·737
	37	—	—	—	8·688
1	40	+0·043	+1·825	8·614	8·616
2	45	+0·152	+1·401	8·5040	8·501
1	50	−0·044	+2·180	8·3852	8·389
2	55	+0·019	+1·892	8·2824	8·281
					$\sigma = 0{\cdot}003$

Table 5. *First dissociation of arginine: thermodynamic quantities*

σ is the standard error (Please, 1954).

Temp.	$\Delta G°$ (kJ/mole)	$\Delta H°$ (kJ/mole)	$-\Delta S°$ (J/mole deg.)	$-\Delta C_p°$ (J/mole deg.)
0°	9·994	6·80	11·7	103
5	10·057	6·28	13·6	105
10	10·130	5·75	15·5	106
15	10·212	5·21	17·4	108
20	10·303	4·67	19·2	110
25	10·404	4·11	21·1	112
30	10·514	3·55	23·0	114
35	10·634	2·97	24·9	116
37	10·685	2·74	25·6	117
40	10·763	2·39	26·7	118
45	10·902	1·79	28·6	120
50	11·049	1·19	30·5	122
σ_0	0·014	0·36	1·3	14
σ_{25}	0·008	0·10	0·4	15
σ_{50}	0·014	0·40	1·3	16

Table 6. *Second dissociation of arginine: thermodynamic quantities*

σ is the standard error (Please, 1954).

Temp.	ΔG^0 (kJ/mole)	ΔH^0 (kJ/mole)	$-\Delta S^0$ (J/mole deg.)	$-\Delta C_p^0$ (J/mole deg.)
0°	50·821	45·81	18·4	35
5	50·914	45·63	19·0	36
10	51·011	45·45	19·6	36
15	51·111	45·26	20·3	37
20	51·214	45·08	20·9	38
25	51·320	44·89	21·6	38
30	51·430	44·69	22·2	39
35	51·542	44·50	22·9	40
37	51·588	44·42	23·1	40
40	51·658	44·30	23·5	40
45	51·777	44·09	24·2	41
50	51·900	43·89	24·8	42
55	52·025	43·68	25·4	42
σ_0	0·013	0·29	1·0	10
σ_{25}	0·007	0·08	0·3	11
σ_{50}	0·013	0·33	1·0	12

values of pK_{1a} and pK_{2a} [pK_a (obs.)] calculated from these equations are given in Tables 3 and 4 respectively. In these tables are also given the values of pK_a calculated from Harned & Robinson's (1940) equation

$$pK = A/T - D + CT. \qquad (11)$$

These are designated pK_a (calc.). The values of the constants of eqn. (11) were obtained by the method of least squares, the data from both sets of observations for each pK_a being combined.

The thermodynamic quantities for both dissociations of arginine have been calculated from the parameters of eqn. (11); they are given in Tables 5 and 6.

DISCUSSION

The values of both pK_{1a} and pK_{2a} for arginine are lower than those for the corresponding ionizations of simple monoamino monocarboxylic acids. Further, the differences between arginine and glycine are constant over a wide temperature range (Fig. 1); similar results are obtained when arginine is compared with α-amino-*n*-valeric acid (Smith, Taylor & Smith, 1937), the pK_a values of which are very close to those of glycine.

Since the magnitude of a pK_a value in aqueous solution is a direct indication of the free-energy change (ΔG^0) on transferring a proton from an acid molecule to a water molecule, a discussion of the differences in pK_a values between acids resolves itself into a discussion of the corresponding thermodynamic quantities.

It is useful to consider the thermodynamic quantities associated with an ionization as arising from two sources: first, the environmental part due to the interaction of the reacting species and the products with the solvent water which we shall

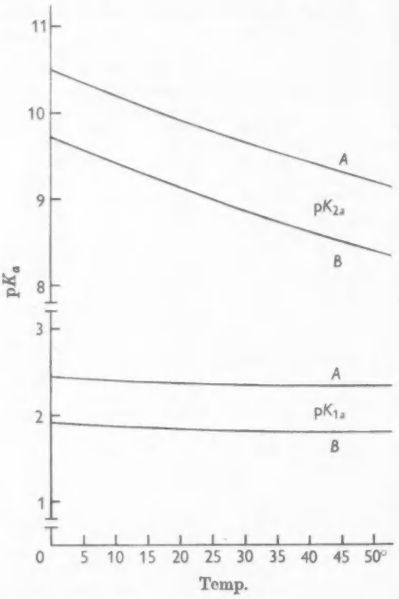


Fig. 1. Temperature-dependence of the thermodynamic pK_a values of glycine (*A*) and arginine (*B*). Values of pK_{1a} for glycine are from King (1951) and of pK_{2a} from Datta & Grzybowski (1958*a*).

term ΔG^0 (env.), ΔH^0 (env.), ΔS^0 (env.) and ΔC_p^0 (env.); secondly, the non-environmental part due to the differences in the binding energy of the proton in the acid and in the hydronium ion (enthalpy) and to the intramolecular rearrangements occurring in the ionization of the acid molecule (entropy).

The latter part also includes a contribution arising from changes in the internal polarization of the molecule which is assumed to be invariant with temperature (cf. the general terms of eqn. 23). These non-environmental parts we shall term ΔG^0 (non.), ΔH^0 (non.) and ΔS^0 (non.). It is likely that ΔH^0 (non.) and ΔS^0 (non.) would remain invariant with temperature, while the environmental parts would reflect the changes in the properties of water with temperature. It follows, therefore, that ΔC_p^0 must arise entirely from environmental factors.

The environmental parts of the thermodynamic quantities are due principally to two effects (Datta & Grzybowski, 1958*b*): (*a*) the electrostatic effect of the reacting ions on the water molecules; (*b*) the stabilization of regions of ice-like structure in the water by large non-polar substituents in the reacting molecules (Frank & Evans, 1945). This latter effect is likely to be important only when the non-polar substituent is very close to the ionizing group, as in the methylammonium ions, sarcosine and *NN*-dimethylglycine; it is unlikely to be important in arginine. We may therefore assume that the electrostatic effect is largely, if not solely, responsible for the environmental parts of the thermodynamic quantities, which may therefore be evaluated from electrostatic theory as follows.

The electrostatic free-energy change on placing an ion, or ions, in solution is inversely proportional to the dielectric constant of the solvent (D); hence, for the ionization of arginine or glycine,

$$\Delta G^0 \text{ (env.)} = p/D, \tag{12}$$

where p is a constant. Consequently, we have the relations:

$$\Delta S^0 \text{ (env.)} = -p[d/dT(1/D)] \tag{13}$$

$$\Delta H^0 \text{ (env.)} = p[1/D - Td/dT(1/D)] \tag{14}$$

$$\text{and } \Delta C_p^0 \text{ (env.)} = \Delta C_p^0 = -p[Td^2/dT^2(1/D)]. \tag{15}$$

The variation with temperature of $1/D$ can be expressed by the relation:

$$1/D = a - bT - cT^2 \tag{16}$$

where, for the experimental values of Malmberg & Maryott (1956),

$$a = 8{\cdot}5756 \times 10^{-3}, \quad b = 2{\cdot}9915 \times 10^{-5}$$

$$\text{and} \quad c = 1{\cdot}4741 \times 10^{-7}.$$

The first and second differentials with temperature of $1/D$ can easily be found from these parameters.

The value of p for any particular ion may be calculated by using an appropriate electrostatic model; the electrostatic contribution to the thermodynamic quantities for a given ionization may be found from a value of p which is the sum of values for the products less the sum of the values for the reactants. Thus for arginine we have

1st ionization:

$$p_{1\,\mathrm{Arg}} = p_{\mathrm{H_3O^+}} + p_{\mathrm{Arg^{2+}-}} - p_{\mathrm{Arg^{2+}}} \tag{17}$$

and 2nd ionization:

$$p_{2\,\mathrm{Arg}} = p_{\mathrm{H_3O^+}} + p_{\mathrm{Arg^{+-}}} - p_{\mathrm{Arg^{2+}-}}. \tag{18}$$

Similarly for glycine:

1st ionization:

$$p_{1\,\mathrm{Gly}} = p_{\mathrm{H_3O^+}} + p_{\mathrm{Gly^{+-}}} - p_{\mathrm{Gly^+}} \tag{19}$$

and 2nd ionization:

$$p_{2\,\mathrm{Gly}} = p_{\mathrm{H_3O^+}} + p_{\mathrm{Gly^-}} - p_{\mathrm{Gly^{+-}}}. \tag{20}$$

The value of $p_{\mathrm{H_3O^+}}$ was obtained from the Born (1920) equation

$$p_{\mathrm{H_3O^+}} = N\epsilon^2/2r_{\mathrm{H_3O^+}} \tag{21}$$

where N is Avogadro's number, ϵ the electronic charge and $r_{\mathrm{H_3O^+}}$ the ionic radius (cm.) of the hydronium ion, taken to be 0·92 Å (Datta & Grzybowski, 1958*b*). The values of $p_{\mathrm{Gly^+}}$ and $p_{\mathrm{Gly^-}}$ were also found from eqn. (21), assuming the ions to be spherical, the radius of the $\mathrm{X{\cdot}CO{\cdot}O^-}$ ion being taken as 1·23 Å (Cohn, 1935) and that of the $\mathrm{X{\cdot}\overset{+}{N}H_3}$ ion as 1·4 Å, the same as that of the ammonium ion (Kapustinsky, 1941). For the glycine dipolar ion, $p_{\mathrm{Gly^{+-}}}$ was found from the equation of Scatchard & Kirkwood (1932), which assumes the molecule to be dumb-bell shaped, the term $1/2r$ in eqn. (21) being replaced by $(1/b - 1/R)$, where b is the radius of each charged group and R is the intercharge distance. The value of b was taken to be 1·315 Å, the mean of the radii of the carboxylate and the ammonium ions, and R was taken as 3·17 Å (Cohn, 1935).

The values of $p_{\mathrm{Arg^{2+}}}$, $p_{\mathrm{Arg^{2+}-}}$ and $p_{\mathrm{Arg^{+-}}}$ were obtained from a modification of Kirkwood's equation (1934), in which the multivalent ion is treated as a sphere of radius b and internal dielectric constant D_i, with point charges $e_1, e_2, \ldots, e_m$ at distances $r_1, r_2, \ldots, r_m$ from its centre; the angle between r_k and r_l is θ_{kl}. The work of charging such an ion at infinite dilution in water is given by:

$$W_0 = \tfrac{1}{2}\sum_{n=0}^{\infty} \frac{(n+1)\,Q_n(D_i - D)}{D_i.b^{(2n+1)}[(n+1)\,D + nD_i]} \tag{22}$$

where $Q_n = \sum_{k=1}^{m}\sum_{l=1}^{m} e_k e_l r_k^n r_n^l \,\mathrm{Pn}(\cos\theta_{kl})$,

in which Pn (cos θ_{kl}) etc. are Legendre functions. Eqn. (22) rearranges to

$$W_0 = \tfrac{1}{2}\sum_{n=0}^{\infty} \frac{(n+1)\,Q_n}{b^{(2n+1)}[(n+1)\,D + nD_i]} - \frac{(n+1)\,Q_n}{D_i.b^{(2n+1)}(n+1+nD_i/D)}. \tag{23}$$

In eqn. (23) the terms involving D_i may be ignored, since they arise from the effect of the electrostatic field on the internal environment of the ion, which should be largely temperature-independent. Thus we obtain the function W_0', which can be used to

find the temperature-dependence of the environmental part of the thermodynamic functions.

$$W_0' = \tfrac{1}{2}\sum_{n=0}^{\infty}\frac{Q_n}{b^{(2n+1)}}\cdot\frac{1}{D}. \qquad (24)$$

Hence p_i (eqn. 12) for any ion is given by

$$p_i = \tfrac{1}{2}\sum_{n=0}^{\infty}\frac{Q_n}{b^{(2n+1)}} = \tfrac{1}{2}z_i^2\sum_{\substack{k=1\\k\neq l}}^{m}\sum_{\substack{l=1\\l\neq k}}^{m}\frac{mbe^2}{b^2-r^2} + \frac{e_k e_l}{b}\frac{1}{\left(1-2\cos\theta_{kl}\dfrac{r^2}{b^2}+\dfrac{r^4}{b^4}\right)^{\frac{1}{2}}} \qquad (25)$$

the assumption being made that $r = r_k = r_l$.

The values of r, b and θ_{kl} for arginine were assigned from measurements of a molecular model (Catalin Ltd., Waltham Abbey, Essex). The distance between any two charges was taken as the mean of the maximum and minimum possible values, and from the three intercharge distances in R^{2+-} the value of r was calculated. The radius of the sphere, b ($b-r$ = charge depth), was computed in such a way that the charge–solvent interaction

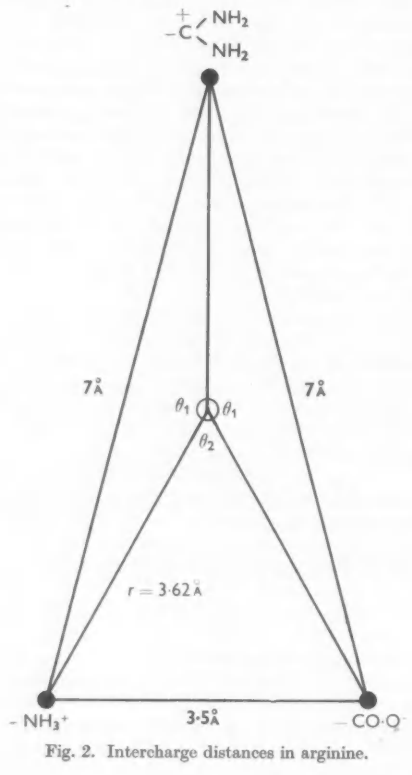


Fig. 2. Intercharge distances in arginine.

Table 7. *Environmental and non-environmental parts of the thermodynamic quantities for the first and second dissociations of arginine and glycine, together with the observed values at 25°*

	$10^{-5}p$	ΔG^0 (env.)	ΔG^0 (non.) (kJ/mole)	ΔG^0 (obs.)	ΔH^0 (env.)	ΔH^0 (non.) (kJ/mole)	ΔH^0 (obs.)	ΔS^0 (env.)	ΔS^0 (non.) (J/mole deg.)	ΔS^0 (obs.)	ΔC_p^0 (env.)	ΔC_p^0 (obs.) (J/mole deg.)
						1st dissociation						
Arginine	7·06	+9·01	+1·39	+10·40	−3·2	+7·3	+4·1	−40·9	+19·8	−21·1	−62	−112
Glycine	9·844	+12·56	+0·86	+13·42	−4·5	+8·5	+4·0	−57·1	+25·5	−31·6	−87	−144
Arginine − glycine	—	−3·55	+0·53	−3·02	+1·3	−1·2	+0·1	+16·2	−5·7	+10·5	+25	+32
						2nd dissociation						
Arginine	4·14	+5·30	+46·02	+51·32	−1·9	+46·8	+44·9	−24·0	+2·4	−21·6	−36	−38
Glycine	7·016	+8·95	+46·86	+55·81	−3·2	+47·4	+44·2	−40·7	+1·8	−38·9	−62	−46
Arginine − glycine	—	−3·65	−0·84	−4·49	+1·3	−0·6	+0·7	+16·7	+0·6	+17·3	+26	+8

p is the electrostatic constant for the various dissociations (eqns. 17–20). The observed values for the first dissociation of glycine are from King (1951) and for the second dissociation from Datta & Grzybowski (1958*b*).

energy, for each charge treated separately, should be the same as for a corresponding singly-charged spherical ion of approximate radius r_i. Thus:

$$\frac{1}{2}\frac{z^2e^2b}{b^2-r^2}\cdot\frac{1}{D} = \tfrac{1}{2}z^2e^2\cdot\frac{1}{r_i}\cdot\frac{1}{D} \qquad (26)$$

hence

$$\frac{b}{b^2-r^2} = \frac{1}{r_i}.$$

The three values of b obtained were averaged. The value of r_i for $-\overset{+}{N}H_3$ and for $-NH-\overset{+}{C}(NH_2)_2$ was taken as 1·40 Å, the same as for the ammonium ion (Kapustinsky, 1941), and for $-CO\cdot O^-$ it was taken as 1·23 Å (Cohn, 1935). From these parameters r for the arginine sphere was calculated to be 3·62 Å (Fig. 2) and b 4·35 Å. For the other species it was assumed that the disappearance of any charge would not alter the relative distance or position of the remaining charges.

The calculated values of p and both the environmental and non-environmental thermodynamic quantities are shown in Table 7. These values show that for the first dissociations of arginine and glycine almost the entire free-energy term arises from environmental factors. When, however, the individual enthalpy and entropy terms are examined, it is seen that there are substantial contributions from the non-environmental parts, but these almost entirely cancel out. The calculated and observed values of ΔC_p^0 do not agree very well but are nevertheless of the same sign. Further, almost the entire difference between the arginine and glycine pK_1 values resides in the entropy terms, the enthalpies being very similar.

For the second dissociations of these amino acids Table 7 shows a great similarity in the observed enthalpy terms, which arise almost entirely from the non-environmental parts. The agreement between the observed and calculated differences in ΔC_p^0 is not, however, quite so good as for the first dissociation. Again, for the second dissociation the major difference in the free-energy terms arises from the environmental parts of the entropy terms.

The implications of these results are: First, that the bond energies of a proton in an amino (or a carboxyl) group in glycine and arginine are virtually identical (non-environmental enthalpy changes). This may well be true for all α-amino acids (Edsall & Wyman, 1958). Secondly, it would appear that the major part of the differences between the dissociations of glycine and arginine is due to differences in the electrostatic interactions of the conjugate acid–base pairs (of different ionic structure) and the solvent water.

SUMMARY

1. The first and second dissociation constants of arginine have been determined over the range 0–55° at 5° intervals, from e.m.f. measurements of cells without liquid junction containing hydrogen and silver–silver chloride electrodes.

2. The standard thermodynamic quantities associated with these dissociations have been calculated. These quantities have been divided into environmental and non-environmental parts.

3. The various factors which contribute to the differences between the first and second dissociation constants of arginine and glycine have been discussed. It is concluded that the pK_{1a} and pK_{2a} values of arginine are lower, by a constant amount, than those of glycine, mainly because of the differences in the electrostatic interactions of the various ionic conjugate acid–base pairs with the solvent water.

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Plant Polyphenols

3. FLAVONOIDS IN GENOTYPES OF *PRIMULA SINENSIS**

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It is now well established that the C_{15} skeleton of the flavonoid molecule is synthesized in plants by the condensation of three acetate units with phenylpyruvic acid (Watkin, Underhill & Neish, 1957). Much less is known about how this skeleton is then elaborated by hydroxylation, methylation and glycosidation. In order to obtain more information about these later stages in biosynthesis, the flavonoids present in several series of colour mutants of cultivated plants are being reinvestigated with recently developed methods of pigment analysis (see Harborne, 1958*a*, *b*, 1959). The results obtained for the diploid potato, *Solanum phureja*, have already been described (Harborne, 1960*a*). The present account is of a similar investigation of the phenolic pigments of *Primula sinensis*, of which many colour mutants of known genetic constitution are available for biochemical analysis (De Winton & Haldane, 1933; De Winton, 1936).

Scott-Moncrieff (1936) and Dayton (1954) have shown that in the inheritance of pigmentation in *P. sinensis*, gene *K* controls the production of malvidin 3-monoside in mauve flowers. Recessive genotypes (*kk*) have coral flowers and contain pelargonidin 3-monoside. Another gene, *Dz*, independently of *K*, also controls production of pelargonidin monoside, though its activity is obscured by complicated dosage and dominance relationships. *KDz* types contain mixtures of malvidin and pelargonidin glycosides and are various shades of red or bluish red; *kDz* types contain pelargonidin 3-monoside only and are bright orange. Gene *R* lowers the pH of the cell sap by 0·6 unit so that *KR* flowers are redder than *Kr* flowers. A gene *B* has a bluing effect on flower colour, by producing flavone copigments, provisionally identified as a mixture of kampferol glycosides. Other genes control pigment distribution; e.g. gene *D* inhibits colour in the flowers. Finally, genes whose main effect is on morphological characters (e.g. leaf shape) may sometimes have minor effects on pigment synthesis.

In spite of all the genetical information available, only one pigment in *P. sinensis* has previously been fully characterized. This is 'primulin', which was isolated from mauve flowers by Scott-Moncrieff (1936) and identified by Bell & Robinson (1934) as malvidin 3-galactoside. That Scott-Moncrieff's pigment must, however, have been the 3-glucoside is shown by the present work, in which 21 glycosides have been identified. They are derived from ten known flavonoid aglycones and all contain glucose and not galactose as their only sugar.

* Part 2: Harborne (1960*b*).

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MATERIALS AND METHODS

Plant material. The 43 genotypes of *Primula sinensis* have been grown under glasshouse conditions from self-seed of diploid lines of plants used in earlier investigations (Scott-Moncrieff, 1936; Dayton, 1954) but also include a few tetraploid lines and some commercial varieties.

Authentic pigments. Known anthocyanins and anthocyanidins were obtained as already described (Harborne, 1958*a*, 1960*a*). Crystalline specimens of primulin, isolated by Miss R. Scott-Moncrieff, were made available by Miss V. C. Sturgess. Kampferol 3-glucoside and 3-rhamnoglucoside were obtained from Professor L. Hörhammer, Munich. Dihydrokampferol (aromadendrin) and its acetate were supplied by Dr R. A. Laidlaw. Kampferol 7-glucoside was prepared from naringenin 7-glucoside (Seikel, 1955) by treatment with alkaline hydrogen peroxide (Simpson & Whalley, 1955) and then with hot dilute acid.

Paper chromatography and spectroscopy. Conditions used for the paper chromatography of phenolic pigments have been described (Harborne, 1958*b*, 1959). The solvent mixtures used were: *A*, butan-1-ol–2N-HCl (1:1, v/v; upper phase); *B*, butan-1-ol–acetic acid–water (4:1:5, by vol.; upper phase); *C*, acetic acid–water (15:85, v/v); *D*, butan-1-ol–benzene–pyridine–water (5:1:3:3, by vol.); *E*, butan-1-ol–ethanol–water (4:1:5, by vol.); *F*, butan-1-ol–water (1:1, v/v; upper phase); *G*, water; *H*, water–conc. HCl (97:3, v/v); *I*, phenol saturated with water.

The spectra of phenolic pigments were determined by the methods of Geissman (1955), Jurd & Horowitz (1957) and Harborne (1958*a*). Measurements were made with a Unicam SP. 500 spectrophotometer.

Extraction of the anthocyanins. Fresh petals or foliar parts were homogenized in methanol containing 1% of conc. HCl. After centrifuging and re-extraction of the residue, the supernatants were combined and evaporated *in vacuo* to a small volume. The concentrate was filtered and the filtrate washed successively with light petroleum and ethyl acetate. When storage was necessary, the extract was first freeze-dried, the product being purified by solution in methanol, followed by precipitation with dry ether; it

was kept in a desiccator and used within 3 months of its preparation.

Separation, purification and identification of the anthocyanins. The methods used have been described by Harborne (1958*a*, *b*, 1960*a*). The anthocyanin concentrates were separated by chromatography on Whatman no. 3 paper with solvent *A*. Successive chromatography in solvents *A*, *B* and *C* of the pigment bands thus obtained eventually gave solutions of pure anthocyanins. Fifteen chromatographically distinct pigments were thus obtained from the genotypes investigated; the best sources of these pigments are given in Table 1. When an anthocyanin was obtained from more than one genotype, identity was established by cochromatography, spectroscopic analysis and examination of the products of acid hydrolysis.

In view of the report of Bell & Robinson (1934) that the major anthocyanin of *P. sinensis* (A13, Table 1) contains galactose, special care was taken in identifying the monosaccharide produced on acid hydrolysis of the anthocyanins. The sugar specimens were inseparable from added glucose, but separated from added galactose on paper chromatograms developed in solvents *B*, *D* or *E* for 36–48 hr. The sugars were oxidized by notatin and also gave the same distinctive colour as glucose when treated with resorcinol in sulphuric acid (Harborne, 1960*a*).

Isolation and identification of the flavonol glucosides. Fresh white petals from plants of Alexandra Beetle *KKDD* genotype were extracted with boiling methanol for 0·5 hr. After filtration, the extract was concentrated *in vacuo* to a small volume and filtered. The pale-yellow extract was separated by chromatography on Whatman no. 3 paper in solvent *B*. The five bands (F1–5; Table 2) were purified by chromatography in solvents *C*, *F* and *G*. Eluates of the two major bands F2 and F4 gave pale-yellow, water-soluble, non-crystalline solids after evaporation to dryness. These were purified further by precipitation with light petroleum from moist butanol. Both compounds retained water of crystallization, even after they had been dried at 100° *in vacuo* for 10 hr. F2 had m.p. 204° (Found: C, 51·3; H, 5·4. $C_{27}H_{30}O_{16}.3H_2O$ requires C, 51·1; H, 5·7%). F4 had m.p. 234–236° (Found: C, 50·6; H, 5·9. $C_{33}H_{40}O_{21}.H_2O$ requires C, 50·1; H, 5·4%). F2 and F4 had the same infrared spectra as known kampferol 3-glycosides (e.g. the 3-glucoside). The other three flavonol glucosides were not present in sufficient amounts for characterization in the above-described manner. The five compounds F1–5 and the aglycones and sugars they give on acid hydrolysis were identified by similar methods to those described (Nordström & Swain, 1953; Harborne, 1959). Sugar:aglycone ratios were determined as described by Harborne (1960*a*). The sugar was also determined by the resorcinol–sulphuric acid reagent (Devor, Conger & Gill, 1958), since the flavonol aglycones do not interfere with the colour produced in this reaction.

Determination of the position of sugar residues of the flavonols. Measurement of the absorption spectra of F1–5 in ethanol and then in ethanol containing inorganic ions (see Table 2) indicated that only the 3- position in these flavonol molecules contains a sugar substituent (see Jurd & Horowitz, 1957). This was confirmed in the following way. The five pigments were fully methylated and then hydrolysed with acid (Nordström & Swain, 1953). The products were then compared with the respective authentic, partially methylated flavonols. 5:7:4′-Trimethylkampferol and 7:4′-dimethylkampferol were prepared as described above from kampferol 3-glucoside and robinin (kampferol 3-rhamnosylgalactoside, 7-rhamnoside). The former substance and the products from F2 and F4 had $\lambda_{max.}$ in 95% ethanol at 257 and 351 mμ, in 95% ethanol–NaOH at 260 and 390 mμ and in ethanol–sodium acetate at 257 mμ; the latter compound had $\lambda_{max.}$ in 95% ethanol at 265 and 338 mμ and in ethanol–sodium acetate at 270 mμ. R_F values and colour of these two compounds have been recorded (Harborne, 1959). The products from F3 and F5 were similarly found to be identical with 5:7:3′:4′-tetramethylquercetin (Herrmann, 1958). The products from F1 and from myricitrin (myricetin 3-rhamnoside) were also identical.

Isolation and identification of sinensin. Fresh orange flowers of Dazzler (*kkDzDz*) genotype were extracted with methanol containing 1% of conc. HCl. After filtration, the extract was evaporated *in vacuo* to an aqueous residue. Alternatively, dried powdered petals were extracted with 0·3N-HCl. In either case, the aqueous solutions were repeatedly extracted with small volumes of ethyl acetate. Evaporation of the ethyl acetate extracts gave a hygroscopic white powder which was purified by chromatography in solvent *G* on Whatman no. 3 paper to give sinensin (R_F 0·72) and an oxidation product (R_F 0·02). After precipitation from a cold methanolic solution with light petroleum, sinensin had m.p. 182° (decomp.). On acid hydrolysis it gave glucose, and an aglycone which, after three recrystallizations from water, separated as colourless needles, m.p. 238–240° (decomp.) and mixed m.p. with dihydrokampferol 236–238° (decomp.) (Found: C, 61·8; H, 4·3. Calc. for $C_{15}H_{12}O_6$: C, 62·5; H, 4·2%). The aglycone and dihydrokampferol had the same R_F values and ultraviolet spectra (see Table 2), and both gave pelargonidin on reductive acetylation (Pacheco, 1957). The structure of sinensin was confirmed by identifying its oxidation product (isolated as described above or from aqueous solutions of sinensin which had been boiled for 4 hr.) as kampferol 7-glucoside (Table 2).

Provisional identification of a derived disaccharide. Chromatograms of the acid hydrolysates of pigments that contained more than one glucose residue (notably A2, F2 and F4) showed the presence, after spraying with aniline hydrogen phthalate, of glucose and a single disaccharide. The same disaccharide was produced by heating the pigments for 4 hr. in 10% (v/v) acetic acid, as was used for preparing rutinose from rutin (Arakawa, 1956). This disaccharide (R_G 0·27, 0·43) was chromatographically identical with gentiobiose in solvents *D* and *B* and separated from added cellobiose (R_G 0·44, 0·58) and maltose (R_G 0·52, 0·58). It was different from the disaccharide (R_G 0·43, 0·61) produced during the acid hydrolysis of pigments isomeric with A2 and A5 which were isolated from *Papaver rhoeas* and related species (see Results).

Survey of the flavonoids. Petal and leaf extracts of all the colour genotypes were examined by one- and two-dimensional paper chromatography. The presence or absence of the 21 major pigments was determined by analysis of R_F values and colours. Acid hydrolysates of all the above-mentioned extracts were similarly examined for the presence or absence of the ten known flavonoid aglycones.

Measurement of pigment concentrations. The centres of the flowers (which contain plastid pigments) were removed by means of a 12 mm. cork borer from fresh corollas (ten per

sample) of the genotype under examination. The samples were weighed within 20 min. of picking and then extracted by crushing them in known volumes of methanol–conc. HCl (97:3, v/v). The extracts were stored for 24 hr. in the dark at 0°. Pigment concentrations were then estimated in the filtered petal extracts by spectrophotometric measurements at the appropriate absorption maxima; the results were compared with similar measurements made on solutions containing known amounts of pure pigment. Total anthocyanin concentration was thus measured at 507 mμ for *kk* types and at 532 mμ for *KK* types. No other pigments were present to interfere with anthocyanin spectra in the visible region. Total flavonol glucoside concentration was measured at the maxima in the 400–420 mμ region after addition of alkali to the petal extract. Interfering substances such as cinnamic acids are virtually absent from *P. sinensis* petals; an alkaline solution of sinensin shows no absorption in this region. Sinensin concentration was measured at its $\lambda_{max.}$ at 287 mμ after it had been separated from other pigments by chromatography in solvent *B* and subsequent elution. Results are given in Tables 3 and 6.

Measurements were also made of the relative amounts of the various pigments on eluates of bands cut from chromatograms developed with solvent *A* or *B*, of fresh extracts, or, in a few cases, of fresh extracts after acid hydrolysis. Results are given in Tables 4 and 5.

RESULTS

Identification of the anthocyanins

Fifteen anthocyanins have been found in flowers and leaves of *Primula sinensis* and some of their properties are listed in Table 1. They all give one of the six common anthocyanidins and glucose on acid hydrolysis. No galactose was detected, in spite of the earlier identification of the major malvidin pigment (A13 in Table 1) as the 3-galactoside (Bell & Robinson, 1934). A specimen of 'primulin', isolated by Scott-Moncrieff (1936), gave malvidin, mixed with petunidin (about 15%), and glucose on acid hydrolysis.

Quantitative acid hydrolyses of pigments A1–3 give pelargonidin and one, two and three molecular proportions of glucose respectively. The spectra and colour of these three pigments are identical and are the same as those of authentic pelargonidin 3-glucoside, but differ from those of known 3:5- and 3:7-glucosides. Furthermore, A3 gives only two intermediate glucosides (A2 and A1) during acid hydrolysis; A2 gives only one (A1) and A1 gives none. Therefore the glucose residues of all three pigments are attached to only one of the four hydroxyl groups of pelargonidin, the one in the 3- position. Similar hydrolysis experiments establish that the remaining pigments are 3-glucosides, 3-glucosylglucosides (3-diglucosides) or 3-glucosylglucosylglucosides (3-triglucosides) of the other five anthocyanidins. Lack of material has prevented the measurement of sugar:aglycone ratios in all 15 substances. However, their chromatographic behaviour (Table 1) is strong evidence that they all have the number of glucose residues assigned to them. All but three out of the 18 possible combinations of six anthocyanidins with three states of glucosidation have been identified.

The identity of pigments A1, A4, A6, A9, A10 and A13 was confirmed by direct comparison with authentic specimens of the six anthocyanidin 3-glucosides. Pigments A2, A5, A7, A11 and A14 are the 3-diglucosides of pelargonidin, cyanidin, peonidin, petunidin and malvidin respectively. Since A2 and A5 are chromatographically different from the pelargonidin and cyanidin 3-diglucosides, known to occur in various *Papaver* spp. (Robinson & Robinson, 1931; Harborne & Sherratt, 1957), these five pigments are new compounds. Acid hydrolysis of A2 and A5 yields, besides glucose, a small quantity of a disaccharide, which appears to be gentiobiose. As the isomeric cyanidin pigment (mecocyanin) from poppy is supposed to be the 3-gentiobioside (Grove, Inubuse & Robinson, 1934), a re-examination of the two poppy anthocyanins was carried out. On hydrolysis they give glucose and a disaccharide, different in its chromatographic behaviour from gentiobiose but similar to cellobiose.

The remaining four pigments, A3, A8, A12 and A15, are the 3-triglucosides of pelargonidin, peonidin, petunidin and malvidin and are also new pigments. The related cyanidin 3-triglucoside has been identified as occurring in acylated form in red cabbage (Stroh, 1959), so that only the delphinidin glycoside of this structure remains to be found.

Identification of the flavonol glucosides and of sinensin

Five main flavonol glucosides occur in flowers of *P. sinensis* and at least three of them are also present in the leaves. Their properties are given in Table 2. They have been identified as the 3-diglucoside (F2) and 3-triglucoside (F4) of kampferol, the 3-diglucoside (F3) and 3-triglucoside (F5) of quercetin and the 3-glucoside (F1) of myricetin. Only the last compound (cannibiscitrin) has been thoroughly described before (see Karrer, 1958): it occurs in the flowers of *Hibiscus cannabinus*, in leaves of tea (*Camellia*) and in the seed-coat of *Phaseolus vulgaris*. The 3-triglucosides of kampferol and quercetin were thought to occur in the leaves of tea (Oshima & Nakabayashi, 1954), but reinvestigation by Roberts, Cartwright & Wood (1956) did not reveal them.

Sinensin (Harborne & Sherratt, 1958) is present in nearly all genotypes of *P. sinensis*, but occurs in quantity in flowers of only a few, e.g. the Dazzler mutant. It has been identified as dihydrokampferol 7-glucoside. Its properties closely resemble those of dihydrokampferol 7-methyl ether (Chopin &

Table 1. *Sources and properties of the anthocyanins of* Primula sinensis

Pigment	Colour in visible (and in ultraviolet) light	Principal source	Visible spectra: $\lambda_{max.}$ in methanol–0·03N-HCl (mμ)	Visible spectra: $E_{440\ m\mu}/E_{max.}$ pigment (as %)	R_F: Solvent *A*	R_F: Solvent *B*	R_F: Solvent *H*	Products of complete hydrolysis	Intermediate products of hydrolysis	Ratios: aglycone / glucose
A1	Orange–red (dull red)	'Dazzler' flowers (*kkbbRRDzDz*)	508	39	0·43	0·48	0·12	Pelargonidin glucose	None	1:1·10
A2			507	39	0·25	0·32	0·21		A1	1:1·82
A3			510	36	0·18	0·26	0·36		A1 and A2	1:3·28
A4	Magenta (dull magenta)	'Dazzler' flowers	520*	29	0·14	0·26	0·07	Cyanidin glucose	None	—
A5		'Dazzler' leaves	521*	29	0·10	0·20	0·14		A4	—
A6		'Dazzler' flowers	518	29	0·16	0·33	0·09	Peonidin glucose	None	—
A7		'Dazzler' leaves	519	33	0·10	0·25	0·19		A6	—
A8		'Dazzler' leaves	518	35	0·09	0·10	0·36		A6 and A7	—
A9	Dull mauve (dull mauve)	'Duchess Fern Leaf' flowers (*KKbbRR*)	534*	18	0·11	0·15	0·03	Delphinidin glucose	None	—
A10		Standard 'stellata' flowers (*KKBBRR*)	534*	20	0·14	0·23	0·03	Petunidin glucose	None	1:1·01
A11		'Beetle' leaves (*KKDD*)	531*	23	0·07	0·16	0·12		A10	—
A12		'Beetle' leaves	533*	24	0·05	0·11	0·29		A10 and A11	1:2·78
A13		Standard 'stellata' flowers	533	26	0·16	0·33	0·04	Malvidin glucose	None	1:1·06
A14		'Beetle' leaves	532	25	0·10	0·19	0·15		A13	—
A15		'Beetle' leaves	532	22	0·06	0·14	0·31		A13 and A14	1:2·79

* Only these pigments had their spectra shifted to the blue region by the addition of ethanolic $AlCl_3$.

Table 2. *Properties of the flavonol glucosides, sinensin and its derivatives*

The aglycones were: F1, myricetin; F2, F4, kampferol; F3, F5, quercetin.

Pigment	$\lambda_{max.}$ (mμ)				
	95% Ethanol	95% Ethanol with 2N-NaOH (2 drops/3 ml.)	95% Ethanol with 5% of $AlCl_3$ (2 drops/3 ml.)	95% Ethanol satd. with sodium acetate	95% Ethanol satd. with H_3BO_3 and sodium acetate
F1	258, 305, 363	Decomp.	365, 398	264	262, 305, 385
F2	267, 298, 349	275, 398	351, 395	274	268, 305, 353
F3	258, 305, 360	Decomp.	367, 388	272	265, —, 385
F4	268, 298, 350	275, 397	275, 393	274	267, 298, 350
F5	257, 305, 361	Decomp.	337, 410	273	265, —, 385
Sinensin	215, 287, ~325	288, 365	309, ~370	—	—
Sinensin acetate	271, ~305	—	—	—	—
Oxidation product of sinensin	265, 325, 366	410 (slow decomp.)	—	264	—
Kampferol 7-glucoside					
Sinensin aglycone	215, 292, ~330	243, 318	314, ~365	—	—
Dihydrokampferol					

Pigment	R_F			Colour in ultraviolet light		Intermediate products of hydrolysis	Ratio: aglycone / glucose
	Solvent *B*	Solvent *G*	Solvent *I*	In air	In NH_3 vapour		
F1	0·47	0·05	0·32	Ochre	Yellow–brown	None	1:0·96
F2	0·43	0·27	0·55	Ochre	Yellow–green	None	1:1·96
F3	0·37	0·19	0·36	Ochre	Yellow	None	1:2·09
F4	0·31	0·33	0·45	Ochre	Yellow–green	F_2	1:3·35
F5	0·23	0·18	0·26	Ochre	Yellow	F_3	1:2·86
Sinensin	0·57	0·64	0·76	Dull purple	Light yellow	None	1:0·95
Oxidation product of sinensin	0·54	0·02	0·76	Bright yellow	Bright yellow	None	1:0·88
Kampferol 7-glucoside							
Sinensin aglycone	0·92	0·30	0·77	Dull purple	Dull purple	—	—
Dihydrokampferol							

Pacheco, 1958) but differ from those of dihydrokampferol itself. This dihydroflavonol typically occurs in the free state or as a 3-glycoside in heartwood of trees (Karrer, 1958) so that its presence as the 7-glucoside in a herbaceous plant is of interest.

Distribution of flavonoids

Hydroxylation of the flavonoids. *KK* genotypes, which have coloured flowers (red, maroon, mauve or blue), contain varying amounts of the 3-glucosides of malvidin, petunidin and delphinidin. Myricetin, quercetin and kampferol glucosides are also present and are the only pigments found in white flowers of *KKDD* genotype. By contrast, *kk* types lack pigments with the 3′:4′:5′-trihydroxylation pattern: in them the anthocyanidins are pelargonidin, peonidin and cyanidin and the flavonols are quercetin and kampferol. Gene *K* therefore controls a simple step in biosynthesis, the introduction of a 5′-hydroxyl group into a precursor common to both anthocyanidin and flavonol pigments. Unlike other known genes with similar effect in other plants (e.g. *P* in *Solanum phureja*, Harborne, 1960*a*), it is incompletely dominant in certain genetic backgrounds (see below and Dayton, 1954).

Anthocyanin concentration: the Dazzler factor. *KKDzDz* plants normally have scarlet flowers and contain the anthocyanins of *KK* forms, together with those of *kk* forms. Plants of constitution *kkDzDz* (orange flowers) differ from *kkdzdz* forms (coral flowers) only by containing more of the same anthocyanins. The concentrations of the three types of pigments of flowers of *kkDzDz* and *kkdzdz* genotypes have been measured and the results are shown in Table 3. Whereas the amount of flavonol glucoside and sinensin present is relatively constant, there is a threefold increase in the amount of pelargonidin glucosides on passing from the recessive to the dominant genotype. The Dazzler gene thus increases the total concentration of anthocyanins, and only anthocyanins, in the flowers. The production in *KKDzDz* plants of pelargonidin, cyanidin and peonidin glucosides, anthocyanins typical of the recessive *kk* forms (not, as might be expected, more malvidin and petunidin 3-glucosides), must be due to the fact that the amount of enzyme available in *KK* types for 5′-hydroxylation is strictly limited.

Methylation of the anthocyanins. There are noticeable differences in the degree of methylation between different genotypes and between different tissues of the same genotype. The proportions of 3-glucosides of delphinidin, petunidin and malvidin in the flowers of a series of *KK* genotypes are recorded in Table 4. The amount of methylation is greatest in the wild-type plant and in six other *BB* forms examined (measurements on only one typical example are given in Table 4). Genotypes recessive for the *b* or copigment gene have 10 % less malvidin present. Methylation is further decreased in some morphological mutants, being, indeed, absent from the 'Duchess Fern Leaf' form. These results suggest that there are genes controlling

Table 3. *Amounts of different pigments in* DzDz *and* dzdz *genotypes*

Results given are the averages of three determinations and are expressed as mg. of pigment/g. fresh wt. of flower petal.

		Concentration in		
Pigment type	Estimated as	*kkDzDz* (orange 'Dazzler')	Pale coral mutant*	*kkdzdz* (coral 'Pink Star')
Anthocyanin	Pelargonidin 3-diglucoside	3·2	1·1	1·0
Flavonol glucoside	Kampferol 3-diglucoside	0·67	0·60	1·7
Dihydroflavonol	Sinensin	7·2	6·1	7·2

* The genotype of this mutant, which is morphologically closer to 'Dazzler' than 'Pink Star', is not known.

Table 4. *Amounts of 3-glucosides of malvidin, petunidin and delphinidin in the flowers of* KK *genotypes*

			Percentage of total anthocyanin as		
	Constitution	Flower colour	Malvidin 3-glucoside	Petunidin 3-glucoside	Delphinidin 3-glucoside
BB	Standard wild-type	Mauve	75 (±2)	25	<1
B	37 tetraploid	Mauve	73	24	4
bb	Oak tongue	Maroon	65	29	5
bbbb	36 tetraploid	Maroon	60	30	7
bb	Fasciated	Maroon	63	28	9
bb	'Sirdar'	Pink	48	52	<1
bb	'Reading Pink'	Pink	13	51	37
bb	'Duchess Fern Leaf'	Blue	0	0	100

methylation in *P. sinensis* and that they interact with other genes.

The expected correlation between an increase in methylation and a reddening effect on flower colour (cf. Scott-Moncrieff, 1936) is not apparent from the data of Table 4. Evidently, methylation as a determinant of colour is masked by other factors, such as copigmentation. The 'methylating genes' are less effective in their action in leaves than in flowers: for example, petunidin is the major anthocyanidin in the leaves of *KK* types, of which the flowers contain 60–75 % of malvidin; again, cyanidin, which is largely masked as a flower anthocyanidin by overwhelming amounts of peonidin and pelargonidin, is the major leaf pigment aglycone in *kk* forms.

Table 5. *Distribution of flavonoid* 3*-glucosides in petals and leaves of* Primula sinensis

Unless indicated otherwise, values are for 3-monoglucoside in petals and for mono-, di- and tri-glucosides in leaves. Results are expressed as percentage of total anthocyanins or flavonol glucosides.

	KK genotypes		*kk* genotypes	
Glucosides of	Petals	Leaves	Petals	Leaves
Malvidin	75*	22†	—	—
Petunidin	25*	78†	—	—
Delphinidin	<1	<1‡	—	—
Peonidin	—	—	9	31§
Cyanidin	—	—	<1	69§
Pelargonidin				
3-Glucoside	—	—‖	54	<1¶
3-Diglucoside	—	—	9	—
3-Triglucoside	—	—‖	28	—¶
Kampferol**	88	25	70††	30††
Quercetin**	9	72	30††	70††
Myricetin	3	3	—	—

* Traces of 3-triglucoside are present in two maroon genotypes ('fasciated' and 'oak tongue').

† 80–85 % of total pigment is present as 3-triglucoside.

‡ Monoglucoside.

§ 6, 19 and 75 % of total pigment are present as mono-, di- and tri-glucoside respectively.

‖ Appreciable amounts are present in certain genotypes, e.g. *KKDzDz*.

¶ Increased amounts (up to 10 %) of these pigments are present in *kkDzDz* forms.

** Di- and tri-glucoside.

†† Approximate values obtained by visual estimation of the intensity of aglycone spot on chromatograms.

Glucosidation of the flavonoids. All but one of the flavonols and anthocyanidins occur as 3-mono- -di- or -tri-glucoside in relative amounts that vary with the situation in the plant (Table 5): generally a higher degree of glucosidation of the anthocyanidins occurs in leaves than in flowers. In flowers of the *KK* series, 3-glucosides predominate; 3-triglucosides of petunidin and malvidin occur exceptionally in traces in flowers of two out of the 15 *KK* genotypes available for examination. By contrast, leaves of *KK* forms contain mainly triglucosides. In *kk* genotypes the flowers contain 63 % of the total anthocyanin as 3-glucoside, whereas the leaves contain 75 % as triglucoside.

Copigmentation. Gene *B*, which has a bluing effect on flower colour, was originally thought (Scott-Moncrieff, 1936) to control the production of specific flavone copigments. Examination of the flavonol glucosides of a variety of *BB* and *bb* genotypes by paper chromatography shows, however, that qualitative differences in pigmentation are not involved. Measurements were therefore made of the amounts of anthocyanin and flavonol glucoside in flowers of plants of two families, 36 and 37, which were known to be segregating for the *B* factor (Table 6). Because of variations in the amounts of both pigments in the flowers of individual plants, the results are expressed as the ratio of the flavonol glucoside concentration to the total anthocyanin concentration. This ratio in mauve flowers is consistently higher than in maroon flowers. Copigmentation causes a shift of 4–5 mμ in the visible maximum of the anthocyanin (mainly malvidin 3-glucoside), when measured on fresh aqueous extracts. Similar shifts were produced when the purified kampferol 3-diglucoside and 3-triglucoside were added separately to an aqueous acid solution of malvidin 3-glucoside. It is therefore a reasonable assumption that these flavonol glucosides act as copigments, although other factors (e.g. metal-ion concentration) may determine their effectiveness in this respect.

Flavonoids and Primula *taxonomy.* Taxonomists place *P. sinensis* in the section *Sinenses* of this genus. The chemical results are consistent with this classification. A striking similarity between all *Primula* species that have been examined is the

Table 6. *Copigmentation in* Primula sinensis

Family number	Genotype*	Colour	No. of plants examined	$\lambda_{max.}$ in 0·3 N-HCl (mμ)	Average ratio: flavonol glucoside / anthocyanin
36	*Kbdzch*	Maroon	5	516	1·1
	KBdzch	Mauve	3	520	2·8
37	*KbdzCh*	Maroon	4	516	1·1
	KBdzCh	Mauve	4	521	4·9

* These are tetraploids: for simplicity, the full complement of gene symbols are omitted, as the plants are homozygous for pigment genes. The *Ch* gene controls a morphological effect in *P. sinensis*.

constant occurrence of anthocyanins which contain glucose as their only sugar. Thus malvidin 3-glucoside has been found in *P. polyanthus* (Scott-Moncrieff, 1930), malvidin 3:5-diglucoside in *P. viscosa* and in *P. integrifolia* (Karrer & Widmer, 1927), malvidin, petunidin and delphinidin 3:5-diglucoside in *P. obconica* (J. B. Harborne, unpublished work) and hirsutidin 3:5-diglucoside in *P. hirsuta* (Karrer & Widmer, 1927). *P. sinensis* also shares with at least two other species its ability to add trisaccharide residues to the 3-hydroxyl group of the flavonols: kampferol and quercetin 3-triglucoside have been found in flowers and leaves respectively of *P. obconica* and the former pigment occurs in flowers of *P. rosea* (J. B. Harborne, unpublished work). There are, however, at least three ways in which *P. sinensis* differs from other members of the Primulaceae. It apparently lacks the ability that plants such as *P. hirsuta* and *P. rosea* possess of both methylating the 7-hydroxyl group and also glucosidating the 5-hydroxyl group of its anthocyanins. And, unlike the other species, it can synthesize a dihydroflavonol 7-glucoside: sinensin appears to be a characteristic pigment of *P. sinensis*.

DISCUSSION

The original observation of Scott-Moncrieff (1936) that gene *K* in *Primula sinensis* controls the production of malvidin, in place of pelargonidin, does not appear to fit the 'one gene–one enzyme' hypothesis, since at least three biochemical processes separate the two pigments. The present study helps to resolve this difficulty. Traces of delphinidin and cyanidin found respectively in *KK* and *kk* genotypes (as glucosides) show that *K* has, in fact, only one biochemical effect on pigment production. It controls the introduction of a 5′-hydroxyl group into a precursor common to both the anthocyanidins and the flavonols. It is thus parallel in its action to *P* in *Solanum phureja* (Harborne, 1960*a*), *O* in *Streptocarpus* (Lawrence & Sturgess, 1957) and *L* in *Impatiens balsamina* (Alston & Hagen, 1958; Clevenger, 1958).

A feature of flower pigmentation in *P. sinensis* is the predominance of compounds containing the 4′-hydroxyl grouping (e.g. pelargonidin, kampferol, dihydrokampferol) or the 3′:4′:5′-trihydroxyl grouping (e.g. malvidin, petunidin). Cyanidin and quercetin, i.e. compounds with the 3′:4′-dihydroxyl grouping, are relatively unimportant. This result fits the thesis of Lawrence & Sturgess (1957) that cyanidin (and quercetin) are most readily produced in the plant and appear in the flowers of more highly evolved plants, such as *Streptocarpus*, only as 'biosynthetic residues'.

The discovery of *KK* genotypes, in which there is variation in the degree of methylation of the petal anthocyanins, indicates that there are genes present for the control of this process. Though yet unidentified in *P. sinensis*, a parallel study has revealed the presence of two such genes controlling the methylation of delphinidin in *P. obconica* (Harborne, 1960*c*).

As in a number of other plants (Harborne, 1960*c*), there is a close biogenetic relationship between the flavonols and anthocyanidins present in *P. sinensis* and their glycosidation pattern. Not only are the sugars attached to the same hydroxyl group of the anthocyanidins and the flavonols, but also parallel mono-, di- and tri-glucosides have been isolated in both series. In contrast, there appears to be no corresponding link between the flavonols and the dihydroflavonol. Indeed, the production of dihydrokampferol, which occurs as the 7-glucoside (sinensin) by direct reduction of kampferol, present as the 3-di- or tri-glucoside, seems unlikely in view of the difference in the glucosidation pattern of the two classes of pigment. Furthermore, there is no evidence that such a reduction occurs earlier in biosynthesis since the dihydroflavonols related to quercetin and myricetin have not been detected. The inheritance of sinensin is independent of the known genes (e.g. *K*) involved in pigment synthesis. Sinensin appears to be present in all the genotypes in traces; only its concentration is affected by the activity of pigment genes.

The presence in the same plant of related flavonoid 3-glucosides, 3-diglucosides and 3-triglucosides suggests that the glucose residues are attached separately one at a time, rather than that preformed di- and tri-saccharides are attached directly in a single step to the pigment molecules. The isolation from wheat germ (Cardini & Yamaha, 1960) of two enzymes which catalyse the synthesis of phenolic mono- and di-glucosides respectively supports this hypothesis. Since the same disaccharide is produced when the anthocyanidin and flavonol 3-di- and 3-tri-glucosides are hydrolysed, it is possible that the same or similar enzymes control the attachment of glucose residues to both flavonols and anthocyanidins. It is generally considered that in most plants anthocyanins and flavones compete directly for a common precursor, so that increased visual pigmentation is normally correlated with decrease of flavone production and vice versa. Indeed, there is evidence that the genes *K* and *B* interact by competing for this common precursor. The Dazzler gene is different in that it increases anthocyanin concentration without affecting the concentration of other flavonoids present. The only other plant known to contain genes with a similar action to *Dz* is *Impatiens balsamina* (Hagen, 1959). The biochemical action of

Dz may be interpreted by assuming that, as Lawrence & Sturgess (1957) have suggested, pigment genes can stimulate increased production of a common precursor; alternatively, it is possible that there is an unlimited amount of precursor available but that the enzyme controlling anthocyanin formation is limiting.

SUMMARY

1. Fifteen anthocyanins derived from six anthocyanidins have been found in flowers or leaves of *Primula sinensis* colour genotypes. They are 3-glucosides, 3-glucosylglucosides or 3-glucosylglucosylglucosides.

2. Five flavonol glucosides have also been found. They are myricetin 3-glucoside and quercetin and kampferol 3-glucosylglucoside and 3-glucosylglucosylglucoside.

3. The only uncommon pigment present, sinensin, has been identified as dihydrokampferol 7-glucoside.

4. The qualitative and quantitative distribution of these pigments in genotypes of *P. sinensis* is described.

5. These results provide information about the genetic control of the hydroxylation, methylation and glucosidation of the flavonoid pigments. The biochemical effects of factors controlling co-pigmentation and anthocyanin concentration are discussed.

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The Intracellular Distribution of Fatty Acids in Rat Liver

THE FATTY ACIDS OF INTRACELLULAR COMPARTMENTS

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A study has been undertaken of the distribution of fats and their constituent fatty acids in the subcellular organelles of the rat-liver cell. A number of investigators have previously studied the fatty acids of rat liver (Kretchmer & Barnum, 1951; Klein & Johnson, 1954; Clement, Clement & Le Breton, 1956; Holman & Widmer, 1959; Dittmer & Hanahan, 1959*a*, *b*), but in all cases the unsaturated fatty acids were measured by ultraviolet spectroscopy or reversed-phase chromatography. These methods do not distinguish individual fatty acids. Gas chromatography (James & Martin, 1952, 1956) of fatty acid esters has made a more complete analysis of fatty acid mixtures possible (see also Paoletti & Grossi, 1960). This technique was used in the studies described in this paper. The results show that although the fatty acids of the nuclei, mitochondria, fluffy layer and microsomes do show differences, the differences are comparatively slight. The layer of supernatant fat, however, shows large differences in fatty acid composition from the rest of the cell fractions and shows similarities in composition to the dietary fat and the adipose tissue. Our results are essentially in agreement with those of Macfarlane, Gray & Wheeldon (1960) obtained in a similar study.

MATERIALS AND METHODS

Animals. These were rats of the Wistar strain, fed on a standard diet of rat cubes supplied by Oxo Ltd. (Medical Department), Southwark Bridge Road, London, S.E. 1. The maker's analysis showed that the cubes contained 3·9% of oil; our analysis showed 3·24% of total lipid extractable by chloroform–methanol and dried at 105° overnight. Loss of some volatile components would be expected under these conditions.

Chemicals. All solvents were of analytical grade except ethanol which was purchased as 'azeotropic'. Diazomethane was prepared from *p*-nitrosomethyltoluenesulphonamide (British Drug Houses Ltd.).

Preparation of the cell fractions. The rats were killed by stunning and decapitation and the livers were transferred to crushed ice. After a few minutes of chilling the livers were passed through the stainless-steel screen of the Fisher mincer (Jouan, Paris) and the pulp produced was weighed. This pulp constitutes the 'liver' for the purpose of this paper. The connective tissue and other material retained by the grid of the mincer (7% of the wet weight) has been ignored. The pulp was homogenized in approx. 2 vol. of ice-cold 0·25 M-sucrose with a stainless-steel homogenizer of the Potter–Elvehjem type, and the homogenate was diluted to 20% with 0·25 M-sucrose. The procedure for isolating the nuclear fraction and the mitochondria was the same as that of Werkheiser & Bartley (1957), but the fluffy layer was retained and washed in the same way as the mitochondrial sediment. The microsomes were isolated from the combined supernatants of the mitochondrial and fluffy layer fractions by centrifuging for 60 min. at 30 000*g* in the no. 20 rotor of the Spinco centrifuge. The layer of fatty material that floated on the surface of the supernatant was removed and extracted separately.

Extraction of lipids. If distribution studies on lipids are to be meaningful, it is essential either that the extraction of the lipids be quantitative or that the material extracted be a representative sample of the lipid. To assess the completeness of extraction a measured sample of the extract was saponified and the methyl esters of the fatty acids were prepared. The amounts of methyl esters obtained were compared with those obtained by directly saponifying the tissue and preparing the methyl esters. In the best extraction procedure about 90% of the lipid was extracted. Even with the relatively poor extractions the proportions of different fatty acids were the same in directly saponified material as in the saponified extract.

Extraction with chloroform–methanol. Usually the homogenized tissue or tissue fraction was suspended in sufficient 0·25 M-sucrose for samples to be pipetted. Samples of liver pulp were weighed directly without homogenization in sucrose. The weighed or measured sample was homogenized in 10 vol. of $CHCl_3$–methanol (2:1, v/v) and kept for 1 hr. After filtering through a fat-free filter paper the solid residue and the filter paper were extracted with a further 10 vol. of the $CHCl_3$–methanol and kept at room temp. for a further 2 hr. The extracts were then combined and subjected to the Folch procedure (Folch, Lees & Sloane-Stanley, 1957). The organic layer was evaporated to dryness and the residue dissolved in $CHCl_3$ containing 2% of methanol. A measured volume of this extract was evaporated to dryness to determine total lipids.

Because of the large volume of the supernatant fraction from the cell fractionation it was impracticable to extract it directly with 10 vol. of $CHCl_3$–methanol mixture. The supernatant was therefore either freeze-dried before extraction with the mixture or the proteins and lipids were precipitated by adding 50% (w/v) trichloroacetic acid to give a final concentration of 10%. The precipitated material

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was washed once with 5% (w/v) trichloroacetic acid and then with water, before extraction with $CHCl_3$–methanol. In one experiment the microsome fraction as well as the supernatant fraction was freeze-dried before lipid extraction and in one experiment all the cell fractions were treated with trichloroacetic acid before extraction. In the latter case the precipitated material was extracted by refluxing in a Soxhlet apparatus for 2 hr. with 100 ml. of $CHCl_3$–methanol (1:1, v/v). The extracted material was evaporated to dryness under a stream of N_2, re-extracted with $CHCl_3$–methanol (2:1, v/v) and subjected to the Folch purification procedure.

Extraction with ethanol–ether. Hanahan, Dittmer & Warashina (1957) have used extraction with ethanol followed by ethanol–ether mixtures to remove tissue lipids. In one experiment their procedure was followed and tissue fractions were extracted with 10 vol. of 95% ethanol at room temp. for 4 hr. The residue was then re-extracted with 10 vol. of ethanol–ether (3:1, v/v) for a further 3 hr. The latter extraction was repeated and the three extracts were combined. After the extracts were dried, the residue was exhaustively extracted with warm light petroleum (b.p. 40–60°). A sample of this extract was dried for determination of total lipid.

Direct saponification. A measured portion of the tissue, tissue homogenate, or tissue fraction (equivalent to not more than 1 g. wet wt. of tissue) was incubated for 3 days at 30° with 5 ml. of methanolic KOH containing quinol (20 ml. of 10N-KOH, 50 ml. of methanol, 0·1 g. of quinol, 30 ml. of water).

Saponification of the lipid extracts. A measured volume of the lipid extract was evaporated to dryness and 5 ml. of saponification mixture was added. The mixture was then kept at 30° for 3 days.

Extraction of the fatty acids. Water (15 ml.) was added to the saponification mixture and then 15 ml. of light petroleum (b.p. 40–60°). After thorough shaking, the solvent layers were allowed to separate and the upper phase was drawn off with a pipette. The extraction was repeated twice with 15 ml. portions of light petroleum. The water layer was then acidified by the addition of 2 ml. of 50% (v/v) H_2SO_4 and extracted three times with 15 ml. portions of the light petroleum.

Preparation of the methyl esters of the fatty acids. Freshly prepared diazomethane in ether was added to the chilled petroleum extracts of the fatty acids [dried by the addition of a mixture of anhydrous Na_2SO_4 and anhydrous $NaHCO_3$ (4:1, w/w)] until the solution remained permanently yellow. After 30 min. the solution was warmed to remove excess of diazomethane and stored at 0° until required for gas chromatography or estimation of methyl esters. In the esterification with methanol the esters were made and purified according to Stoffel, Chu & Ahrens (1959). In all cases the organic solvent was removed from the methyl esters by evaporation under a stream of N_2.

Gas chromatography. All samples were chromatographed on two different columns. Two chromatographs were used simultaneously. One was a Pye Argon chromatograph (Pye Ltd., Cambridge) and the other similar apparatus was built in this laboratory with the assistance of Mr A. Renshaw. The design for column and detector, which used a 10 mi. strontium foil, was kindly provided by Dr J. E. Lovelock (see Lovelock, 1958). The amplifier was a simple, single-valve (Osram ET/3) amplifier kindly supplied by Mr A. Thomson of the Dyson Perrins Laboratory, Oxford. The recorder was a Honeywell Brown 2 mv full-scale potentiometric recorder. The Pye Argon chromatograph housed a column packed with 20% Apiezon L on Embacel (May and Baker Ltd.) sieved to 90–100 mesh. The argon flow rate was 50 ml./min. and the temp. (200°) was maintained by constant voltage heating. This column gave good recoveries of standard mixtures, which included methyl linoleate (California Foundation) and was used as the reference column throughout this study. The other machine housed a column 4 ft. long and 4 mm. in internal diameter, packed with acid-washed Celite (100–120 mesh) coated with 15% polyethylene glycol adipate ester. The ester was prepared, on the advice of Dr A. T. James, by heating together ethylene glycol and adipic acid in approximately equimolar quantities for 2 hr. at 180° in the presence of a small quantity of *p*-toluenesulphonic acid as a catalyst. The column-packing material, after coating, was resieved to 100–120 mesh before packing. The column was preheated in a stream of argon for about 72 hr. before use for analysis and was operated at 175° with a flow rate of argon of about 60 ml./min. The complementary data from chromatographic analyses on these two columns were combined by using ratios of single components common to both. Major acids were identified by comparison with the retention times of pure standards, all times being measured from the air peak. Some other acids were tentatively identified by comparison of their retention times, relative to methyl stearate, with those published in the literature, e.g. Insull & Ahrens (1959). Where the two chromatograms were in disagreement, as they occasionally were for the unsaturated esters, reliance was placed on the results of the Apiezon column, since the polyester columns sometimes underestimate these acids (Orr & Callen, 1959). The exception to this is methyl oleate which was sometimes difficult to assess accurately on the Apiezon column owing to its poor resolution from methyl linoleate. The fatty acid notation is that suggested by Ahrens *et al.* (1959). Thus oleic acid is designated 18–1 and arachidonic acid 20–4, indicating the numbers of double bonds without indicating their position.

Determination of dry weight of tissues. Samples of the tissues, homogenates, or tissue fractions were treated with 10% (w/v) trichloroacetic acid. The precipitated protein and lipid were treated according to Werkheiser & Bartley (1957).

Measurement of fatty acid esters. This was according to Rapport & Alonzo (1955).

RESULTS

Efficiency of the extraction procedures

When extractions of lipids by three different methods (by chloroform–methanol, ethanol–ether and chloroform–methanol after trichloroacetic acid treatment) from liver pulp were compared large differences were found. Because of the variation, a more detailed investigation of lipid extraction from liver pulp and mitochondria was made. Table 1 compares the amounts of 'lipid' extracted directly from the tissue with that extracted from the tissue homogenized in 0·25M-sucrose. Whereas there is no difference in the amount of 'lipid' extracted when chloroform–methanol was used

Table 1. *Extraction of lipids from liver pulp and mitochondria*

For extraction procedures see text.

Tissue fraction	Extraction method	Weight of 'lipid' extracted/g. of wet tissue or/ml. of mitochondrial suspension (g.)	Methyl esters formed after extraction and saponification (μmoles/g. dry wt.)
Rat-liver pulp	Chloroform–methanol	0·0406	63·0
Rat liver homogenized in 0·25 M-sucrose	Chloroform–methanol	0·0411	63·4
Rat-liver pulp	Ethanol–ether	0·0576	86·0
Rat liver homogenized in 0·25 M-sucrose	Ethanol–ether	0·0816	85·0
Rat-liver mitochondria	Chloroform–methanol	0·0063	6·7
Rat-liver mitochondria	Ethanol–ether	0·0072	8·9

Table 2. *Comparison of percentage composition of fatty acids of liver homogenates as shown by direct saponification of the tissue and by saponification after extraction with chloroform–methanol* (2:1)

For details of extraction procedure see text. The percentages are calculated on a molar basis. br., Branched chain.

Fatty acid	Percentage of fatty acid present after	
No. of C atoms and double bonds	Direct saponification	Extraction
14–0 (myristic)	0·64	0·68
15–0	0·63	0·61
16–0 (palmitic)	19·1	21·6
16–1 (palmitoleic)	Present	Present
17–0 (br.)	Present	Present
18–0 (stearic)	19·1	17·7
18–1 (oleic)	19·7	19·1
18–2 (linoleic)	24·3	24·0
20–4 (arachidonic)	13·6	13·4
22–6	3·25	3·09
Unidentified compound	3·64	3·51

Table 3. *Total lipid content of rat liver and rat-liver subcellular fractions*

Mean values for total lipids extractable by exhaustive extraction.

Fraction	Lipid as percentage of dry wt. of tissue fraction	Fatty acid esters/g. dry wt. of tissue fraction (μmoles)
Liver pulp	23	363
Nuclei	15·8	265
Mitochondria	23·4	384
Fluffy layer	24·3	464
Microsomes	30·9	525
Supernatant	11·2	106

there was, apparently, an increased extraction from the homogenate in sucrose when ethanol–ether was used. This increase was shown to be partly due to extraction of sucrose.

The ethanol–ether extractions did, however, extract appreciably more lipid than the chloroform–methanol as shown by the larger amounts of the methyl esters of the fatty acids that could be prepared from extracts made with ethanol–ether. Similar results were obtained with mitochondria. The differences could be up to 25% of the lipid found. Usually about 90% of the esters shown to be present by the direct saponification technique could be recovered in the extracted lipids when either chloroform–methanol or ethanol–ether was used for extraction. Whereas the precipitation of the lipids of the supernatant fraction did not result in low recoveries, trichloroacetic acid treatment of the particulate fraction or freeze-drying of the sucrose suspension before extraction resulted in recoveries as low as 60%.

Some 5–10% of the lipid of rat liver is very resistant to extraction by the established procedures, but despite variability in the amount extracted there was no indication that lipids extracted did not constitute a representative sample. This is illustrated in Table 2. Similar observations were made on the isolated subcellular fractions. Sex, age or weight of the rats had no obvious effect on the composition of the fatty acid mixture extracted from the liver.

Total lipids of the cell fractions

From the preceding section it is clear that the amount of lipid found in a tissue will depend on the extracting procedure. In Table 3 are given the mean values for the total lipids extractable from the tissue or tissue fraction by exhaustive extraction with chloroform–methanol or ethanol–ether. Of the cell organelles the microsomes contained the highest and the nuclei the lowest proportions of fat.

Changes in the fatty acid composition of homogenates during incubation

Table 4 shows that there was no appreciable change in the fatty acid composition of a rat-liver homogenate incubated for 2 hr. at 37°. It is unlikely, therefore, that the composition of the fatty acids would have changed much in the tissue fractions during the time taken to isolate them from the homogenate. It is, however, possible that

some redistribution of fatty acids may have occurred either between the lipids or the cell organelles.

Fatty acids of the cell fractions

Table 5 presents the mean fatty acid composition of the whole tissue and its constituent cell fractions. The acids in the table comprise more than 95% of the total acids found (trace acids are omitted). No marked differences were seen between the fatty acid distributions in the cell fractions except in the fatty supernatant layer. Of the particulate fractions the mitochondria have the highest concentration of polyunsaturated acids and the lowest oleic and palmitic acid content.

Comparison of the fatty acid composition of the diet, the adipose tissue and the fatty supernatant

Table 6 shows a comparison of the fatty acid composition of the fatty supernatant with that of the dietary fat (rat cubes) and the rat inguinal adipose tissue. They all exhibit low stearate and high oleic and linoleic acid contents. They also have significant amounts of linolenic acid which is present in the diet in rather higher concentration. On the other hand, the fatty supernatant has much higher content of polyunsaturated acids, arachidonic (20–4) and docosahexaenoic (22–6), than the diet and the adipose tissue. In this respect, it is closer to the composition of the rat-liver homogenate. Adipose tissue fat is predominantly triglyceride in nature, and so is that of the diet.

Table 4. *Comparison of percentage fatty acid composition of liver homogenate before and after incubation*

Extraction with chloroform–methanol (see text). The percentages are calculated on a molar basis.

No. of C atoms and double bonds	Homogenate	Homogenate after 2 hr. incubation
14–0 (myristic)	0·82	0·86
15–0	0·81	0·80
16–0 (palmitic)	28·3	28·0
16–1 (palmitoleic)	2·63	2·38
17–br.	—	0·84
17–0	1·14	0·77
18–0 (stearic)	16·0	16·8
18–1 (oleic)	19·5	20·3
18–2 (linoleic)	15·9	14·9
20–4	11·7	10·3
20–3	—	Present
22–6	3·34	4·18

DISCUSSION

The similarity of particulate fatty acid composition shown in this work is of interest. If particle-bound fat is related to the activities of the individual cell particles, it is reasonable to expect the fat to be more specific to the particular organelle, and to reflect in its composition the differences which are well known to exist in, for example, microsomal and mitochondrial function. Both Clement *et al.* (1956), using rats, and Kretchmer & Barnum (1951), using mice, showed larger differences between the fatty acid content in the different organelles. Both these groups of workers found a higher content of arachidonic than of linoleic acid in the mitochondria and microsomes, the reverse of what we have observed in the present study. The former workers showed a higher content of unsaturated acids in the microsomes than mitochondria, whereas Kretchmer & Barnum also found, as we have done, that the mitochondrial fatty acids are the more highly unsaturated. In both these former studies, diets different from ours may have been used, and the animals used were different.

In one of the groups of rats used by Klein & Johnson (1954) very large concentrations of triene

Table 5. *Mean percentage composition of fatty acid of rat liver and rat-liver subcellular fractions*

The numbers in parentheses are the numbers of experiments. Fatty acids present in trace quantities only are not given. The percentages are calculated on a molar basis.

No. of C atoms and double bonds	Pulp (4)	Nuclei (4)	Mitochondria (5)	Fluffy (3)	Microsomes (4)	Supernatant (4)	Fatty supernatant (2)
14–0 (myristic)	0·70	0·50	0·41	0·44	0·47	0·79	1·41
15–0	0·65	0·57	0·47	0·59	0·53	0·68	0·78
16–0 (palmitic)	24·6	25·7	21·4	21·7	23·4	22·7	24·2
16–1 (palmitoleic)	2·46	1·66	1·63	1·25	1·27	2·34	4·19
17–0	0·83	0·64	0·74	0·84	1·01	0·74	0·31
18–0 (stearic)	13·5	16·4	17·5	17·1	19·9	13·2	1·81
18–1 (oleic)	16·1	14·0	12·2	13·2	13·7	21·6	29·5
18–2 (linoleic)	20·2	16·5	20·6	17·7	16·0	18·7	28·1
20–3	1·07	0·92	1·84	1·10	1·30	0·89	0·27
20–4 (arachidonic)	11·9	14·1	15·4	16·3	15·4	10·3	2·03
22–5	0·90	0·64	0·77	0·93	0·78	0·46	0·95
22–6	4·33	5·96	5·12	5·15	4·73	2·46	2·04
Total unsaturated acids	57·0	53·8	57·5	55·6	53·1	56·8	67·1
Polyunsaturated acids	38·4	38·1	43·7	41·2	38·2	32·9	33·4

Table 6. *Comparison of the percentage fatty acid composition of the dietary fat with that of the adipose tissue, the 'floating supernatant' and the liver homogenate*

Extraction with chloroform–methanol (see text). The percentages are calculated on a molar basis.

No. of C atoms and double bonds	Food: rat cubes (5 g.)	Rat inguinal adipose tissue (single rat)	Floating fatty supernatant (pooled from 8 rats)	Rat-liver homogenate (single rat)
9–br.	—	0·14	—	0·05
12–0 (lauric)	—	0·056	0·060	0·06
13–0	—	—	—	0·08
13–br.	—	—	—	—
14–0 (myristic)	0·42	0·99	1·1	0·82
14–1 (myristoleic)	—	—	—	0·04
15–0	—	0·296	0·56	0·50
15–br.	—	0·081	—	0·21
16–0 (palmitic)	18·2	23·2	26·4	25·00
16–1 (palmitoleic)	1·0	3·00	2·86	1·9
16–br.	0·32	0·42	—	—
17–0	—	0·368	0·39	0·25
17–br.	—	0·217	0·31	—
18–0 (stearic)	1·59	2·89	2·02	11·9
18–1 (oleic)	29·9	35·2	28·1	20·5
18–2 (linoleic)	40·5	31·2	27·1	22·8
18–3 (linolenic)	3·1	0·290	0·56	—
19–br.	—	0·256	0·66	—
19–0	1·2	—	—	—
20–0	—	—	0·55	—
20–1	1·25	0·678	0·76	0·30
20–2	—	—	0·45	—
20–3	0·61	—	0·55	0·20
20–4 (arachidonic)	0·86	0·617	1·98	9·80
20–5	—	—	0·51	0·54
21 unsaturated	—	—	0·83	—
22–6	1·07	0·119	3·13	3·71
22–5	—	0·052	1·39	0·50
24 unsaturated	—	—	0·56	0·67
Total unsaturated acids	78·29	71·16	68·78	60·92
Polyunsaturated acids	46·18	32·28	35·11	37·55

acids appeared in the older animals, which they thought to be due to the diet being marginally deficient in essential fatty acids. The concentration of eicosatrienes (20–3) was not remarkably high in any of our analyses.

The arachidonate/linoleate ratio is highest in the microsomes in this study and it has been established that linoleate gives rise to arachidonate *in vivo* (Steinberg, Slaton, Howton & Mead, 1956). This suggests the possibility that the microsome enzymes may be involved in the transformation of linoleate to arachidonate.

The fatty acid composition of the fluffy layer as compared with that of mitochondria and microsomes is consistent with the conclusion, but does not by any means prove, that the fluffy layer is a mixture of mitochondria and microsomes.

SUMMARY

1. The fatty acid composition of rat-liver homogenates and subcellular fractions prepared by differential centrifuging has been measured.

2. Complete extraction of all lipid material from the liver was not achieved by the standard extraction procedures. The proportions of individual fatty acids in the extracted lipids were the same irrespective of the percentage of lipid extracted (60–90%). The fatty acids liberated by direct saponification of the tissue were in the same proportions as in the extracted lipids.

3. The proportions of fatty acids in a rat-liver homogenate remained constant during a 2 hr. aerobic incubation at 37°.

4. The mean fatty acid content (expressed as μmoles of fatty acid/g. dry wt. of tissue) were: liver pulp 363, nuclei 265, mitochondria 384, fluffy layer 464, microsomes 525, supernatant 106.

5. The fatty acid compositions of the different subcellular fractions were very similar. The main fatty acids were palmitic, stearic, oleic, linoleic, arachidonic and docosahexaenoic acid. Mitochondria contained the highest proportion of polyunsaturated fatty acids (43·6%).

6. The fatty acid composition of the supernatant fat after removal of particulate material was similar to that of the food, and similar to that of the adipose tissue.

7. The implications of the results are discussed.

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Determination of Inorganic Sulphate in Studies on the Enzymic and Non-Enzymic Hydrolysis of Carbohydrate and Other Sulphate Esters

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During studies on the hydrolysis of potassium glucose 6-*O*-sulphate by hydrazine (see Dodgson & Lloyd, 1961) it became necessary to develop a new method for determining small amounts of inorganic sulphate. Previous methods developed in these laboratories have involved the use of benzidine (Dodgson & Spencer, 1953; Spencer, 1960) or barium chloranilate (Lloyd, 1959) but cannot be used in the presence of hydrazine. In a previous study of the non-enzymic hydrolysis of crude samples of glucose monosulphate (Dodgson & Spencer, 1954), liberated sulphate was estimated gravimetrically as barium sulphate, but the method was not satisfactory because of the relatively large amounts of substrate which had to be used.

The author's attention was directed (Dr B. Sörbo, personal communication) to the possible use of a turbidimetric method for the determination of inorganic sulphate, gelatin being used as a cloud stabilizer. A method of this type, based on that of Gassner & Friedel (1956), has been developed by Berglund & Sörbo (1959) for the determination of inorganic sulphate in blood and urine. The present paper shows how the method was adapted to the study of the non-enzymic hydrolysis of simple carbohydrate sulphates. Other modifications are described which enable the method to be used, under certain circumstances, for the assay of glycosulphatase and for the microanalysis of ester sulphates.

MATERIALS

Trichloroacetic acid. This was British Drug Houses Ltd. AnalaR reagent.

Barium chloride–gelatin reagent. Micromethods which depend on precipitation of SO_4^{2-} ions in one form or another generally require a certain minimum amount of

SO_4^{2-} ions to be present before precipitation becomes quantitative (see Dodgson & Spencer, 1957; Spencer, 1960). The presence of SO_4^{2-} ions in commercial gelatin preparations ensures that this requirement is fulfilled in the present case. Some gelatins which were tested contained excessive amounts of SO_4^{2-} ions; however, Difco Bacto Gelatin (Difco Laboratories Inc., Detroit, Mich., U.S.A.) proved to be satisfactory in all respects.

The reagent had to be prepared with some care. Gelatin (2 g.) was dissolved in 400 ml. of hot water (60–70°) and allowed to stand at 4° for at least 6 hr. and preferably overnight. Barium chloride (2 g.) was dissolved in the semi-gelatinous fluid and the resultant cloudy solution was allowed to stand for 2–3 hr. before use. Strict adherence to this procedure gave a stabilized suspension of $BaSO_4$ in the presence of excess of $BaCl_2$, which, when mixed with 4% trichloroacetic acid in the proportions 1:4 (v/v), gave an extinction (E) of approx. 0·15 when measured spectrophotometrically in 2 cm. quartz cells at 360 mμ against a water blank. The reagent was stored at 4° and could be used for about 1 week.

Glassware. Glassware was cleaned with HNO_3; mixtures containing H_2SO_4 were avoided. Micropipettes were of the Lang–Levvy constriction type (Lowry, Roberts, Leiner, Wu & Farr, 1954).

Spectroscopic measurements. These were made with the model H 700.308 Uvispek spectrophotometer. A suitable metal slit-mask was constructed to enable measurements to be made in 2 cm. quartz cells with 2 ml. samples (see method B), and the Uvispek quartz 1 cm. microcells and adaptor were used in method C.

Sulphate esters. Monosaccharide mono- and di-sulphate esters, shark chondroitin sulphate and ox chondroitin sulphates A and C were prepared by methods described by Dodgson & Lloyd (1961). The *O*-sulphate esters of serine, threonine and hydroxyproline, and a number of alkyl-sulphates, were prepared according to the methods of K. S. Dodgson, A. G. Lloyd & N. Tudball (unpublished work). Seaweed polysaccharide sulphates were purified and fractionated as described by Lloyd, Dodgson, Price & Rose (1961). Arylsulphate esters were prepared by the general methods outlined by Dodgson & Spencer (1957). Other sulphate esters were gifts.

EXPERIMENTAL AND RESULTS

Determination of sulphate in aqueous solutions

Three different modifications of what was essentially the same method were used. Method A, covering the range 0–200 μg. of SO_4^{2-} ions, was subsequently used to follow the rates of hydrolysis of carbohydrate sulphates by hydrazine (see Dodgson & Lloyd, 1961); method B (range, 0–40 μg. of SO_4^{2-} ions) was later used in enzyme studies on glycosulphatase (see Dodgson, 1961); method C (range, 0–12 μg. of SO_4^{2-} ions) was used in the analysis of various sulphate esters.

Method A. To the sulphate-containing solution (0·2 ml., contained in a tapered 10 ml. centrifuge tube) was added 3·8 ml. of 4% trichloroacetic acid followed by 1 ml. of the $BaCl_2$–gelatin reagent. After mixing, the whole was allowed to stand for 10–20 min. at room temperature. A reagent blank was prepared in the same way except that 0·2 ml. of water was substituted for the sulphate-containing solution. The extinction of the test solution was measured (against the reagent blank) in 2 cm. quartz cells at 360 mμ and a slit width of 0·2 mm. The cloud was stable for at least 1 hr., after which there was some tendency for $BaSO_4$ to settle. A calibration curve was prepared with solutions of K_2SO_4 containing between 20 and 200 μg. of SO_4^{2-} ions (Fig. 1). A second calibration curve was obtained by making spectrophotometric readings at 500 mμ with a slit width of 0·1 mm. (Fig. 1). Greater sensitivity was achieved by reading at 360 mμ, but the 500 mμ calibration curve can be used when estimating SO_4^{2-} ions in the presence of materials tending to absorb ultraviolet light.

Method B. Method A was modified to enable smaller volumes of starting material to be used with increased sensitivity. The sulphate-containing solution (0·1 ml.), contained in a 8 cm. × 1 cm. Pyrex test tube, was mixed

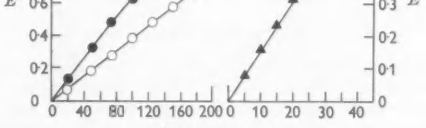

Fig. 1. Calibration curves for the determination of inorganic sulphate by methods A and B (see text). ●, Method A, measurements made in 2 cm. quartz cells at 360 mμ; ○, method A, measurements made in 2 cm. quartz cells at 500 mμ; ▲, method B, measurements made in 2 cm. quartz cells at 360 mμ with the aid of a slit mask.

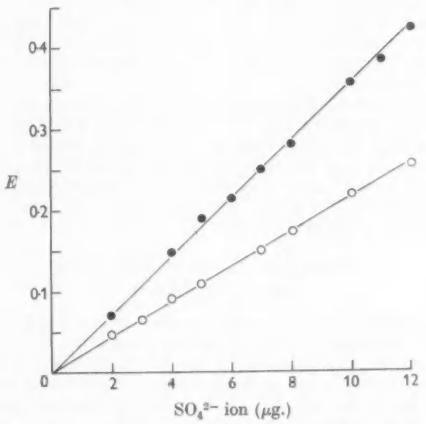

Fig. 2. Calibration curves for the determination of inorganic sulphate by method C (see text). ●, Measurements made in 1 cm. microcells at 360 mμ; ○, measurements made in 1 cm. microcells at 500 mμ.

with 1·4 ml. of 4% trichloroacetic acid followed by 0·5 ml. of $BaCl_2$–gelatin reagent. After the solution was mixed and allowed to stand as before, the extinction was measured (against an appropriate reagent blank) in 2 cm. cells at 360 mμ. Metal blocks (1 cm. high) were inserted in the cell-carrier in order to raise the position of the cells in relation to the light path. A metal plate (16 cm. × 5 cm. × 0·1 cm.), containing a hole (0·5 cm. × 0·5 cm.) suitably positioned to permit the passage of the beam of ultraviolet light, was placed between the cell carrier and the exit slit. The side flanges and two protruding screws which are already present in the cell compartment of the Uvispek provided a convenient means of securing the slit mask in position. All metal surfaces were painted black. A calibration curve was prepared as before in the range 0–40 μg. of SO_4^{2-} ions (Fig. 1).

Method C. This was essentially method B decreased in scale to one-fifth, the determinations being made in 50 mm. × 6 mm. Pyrex tubes. Spectrophotometric readings (at 360 mμ or 500 mμ) were made with the Uvispek 1 cm. microcells, the slit height of the microcell adaptor being adjusted to 4 mm. No 2 cm. microcells were available, consequently the readings were only half as sensitive as those in method B. However, up to 12 μg. of SO_4^{2-} ions could be measured with reasonable sensitivity (Fig. 2).

Effect of varying experimental conditions

Except where otherwise stated, these studies were carried out with method A. Control determinations, in which water was substituted for the solution of K_2SO_4, were made in each case, both test and control solutions being measured against a reagent blank. Generally speaking, control and reagent-blank readings were identical within the limits of experimental error.

Trichloroacetic acid concentration. Calibration curves obtained in presence of 2, 3 and 4% of trichloroacetic acid were identical. At concentrations below and above these limits (1, 5 and 6%) the results tended to be variable and less reliable. A concentration of 4% trichloroacetic acid was selected for general use because of the need to counteract the effect of buffers which were present in later experiments.

Concentration of barium chloride–gelatin reagent. The concentration of this reagent was varied by adding different volumes of the reagent to the trichloroacetic acid–K_2SO_4 mixture and adding appropriate volumes of water to give a final volume of 5 ml. In the one case where the volume of the reagent added exceeded 1 ml. (1·2 ml.), volume compensation was achieved by adding 3·6 ml. of the 4% trichloroacetic acid. Decrease of the volume of added reagent below 0·8 ml. resulted in increasingly poor recoveries of sulphate (Table 1).

Effects of various compounds on the recovery of sulphate

Buffers. Calibration curves for recovery of added K_2SO_4 in the presence of sodium acetate–acetic acid (M), 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–acetic acid (0·5M), Na_2HPO_4–NaH_2PO_4 (0·2M) and sodium citrate–citric acid (0·5M) buffers of varying pH, were identical with that obtained in the absence of buffers.

Hydrazine and imidazole. Solutions of hydrazine hydrate in 0·5M-sodium acetate–acetic acid buffer at varying pH, and solutions of imidazole in the same buffer, did not affect recoveries of added K_2SO_4 (Table 2).

Carbohydrate sulphate esters. Recoveries of K_2SO_4 from buffered and aqueous solutions of various monosaccharide sulphate esters were good (Table 3). With the exception of tripotassium galactose trisulphate, all the esters were stable at room temperature in the presence of trichloroacetic acid for several hours. Galactose trisulphate (prepared by direct sulphation of galactose with excess of chlorosulphonic acid at 70°; Dr A. G. Lloyd, unpublished results) was rapidly hydrolysed by trichloroacetic acid at room temperature. Polysaccharide sulphates formed a complex with the gelatin in the reagent (irrespective of whether $BaCl_2$ was present or not) to give dense cloudy solutions. Berglund & Sörbo (1959) have previously noted that heparin interferes with this type of turbidimetric determination.

Table 1. *Effect of variation of the concentration of barium chloride–gelatin reagent on recoveries of potassium sulphate from aqueous solutions*

Vol. of $BaCl_2$–gelatin reagent present in final vol. of 5 ml. (ml.)	SO_4^{2-} ion (μg.)	
	Added	Recovered
1·2	120	119
	40	39
1·0	200	200
	100	100
	20	20
0·8	200	194
	100	96
	20	19
0·6	200	188
	100	93
	20	18
0·4	200	171
	100	88
	20	15

Table 2. *Effect of hydrazine and imidazole on the recovery of potassium sulphate from solutions buffered with 0·5M-sodium acetate–acetic acid*

	pH	SO_4^{2-} ion (μg.)	
		Added	Recovered
Hydrazine hydrate (0·33M)	4·2	50	50
		100	102
	5·3	50	51
		100	101
	6·8	50	50
		100	102
Imidazole (0·20M)	5·0	25	25
		50	52
		200	199
	6·0	25	25
		50	51
		200	198
	7·5	25	25
		50	51
		200	194

Other sulphate esters. Method B was used for these experiments, the test solutions being measured directly against controls in which water was substituted for the K_2SO_4 solution. Good recoveries were obtained in most cases (Table 4), exceptions being holothurin (a sulphated steroid saponin from sea cucumbers; Nigrelli, Chanley, Kohn & Sobotka, 1955), sodium cortisone 21-sulphate, sodium dehydro*iso*androsterone sulphate (DHAS) and sodium 2-(2:4-dichlorophenoxy)ethyl sulphate (Crag herbicide, see Vlitos, 1953). Holothurin formed an insoluble complex with gelatin, and in the remaining cases recoveries of SO_4^{2-} ions were high. All the sulphate esters tested were stable at room temperature in the presence of trichloroacetic acid during the time of the experiment.

Table 3. *Effect of various carbohydrate sulphate esters on the recoveries of potassium sulphate from aqueous solutions or solutions buffered with sodium acetate–acetic acid (acetate) or tris–acetic acid*

Chondroitin sulphates A and C, shark chondroitin sulphate, charonin sulphate, fucoidin, *Chondrus ocellatus* mucilage and κ- and λ-carrageenins from *C. crispus*, *Gigartina stellata* and *G. radula*, at concentrations of 0·1% in water, all interfered with the method by forming insoluble complexes with gelatin.

Sulphate ester (K^+ or Na^+ salt)	Concn. (mM)	Buffer	Concn. of buffer (M)	pH	SO_4^{2-} ion (μg.) Added	Recovered
Glucose 3-*O*-sulphate	80	Tris–acetate	0·5	5·7	20	20
	50	(Water)	—	—	20	20
Glucose 6-*O*-sulphate	80	Tris–acetate	0·5	5·7	20	21
	80	Acetate	0·5	5·8	20	20
	80	(Water)	—	—	20	20
Glucose disulphate	50	(Water)	—	—	40	42
	50	(Water)	—	—	100	104
Galactose 6-*O*-sulphate	80	Tris–acetate	0·5	5·2	20	20
	25	Acetate	0·25	5·3	40	41
Galactose disulphate	50	Acetate	0·25	5·3	40	39
Galactose trisulphate	50	Acetate	0·25	5·3	40	*

* Rapidly hydrolysed in 4% trichloroacetic acid.

Table 4. *Effect of various sulphate esters on the recoveries of potassium sulphate from solutions buffered with 0·5 M-tris–acetic acid*

Sulphate ester (K^+ salt)	Concn. (mM)	pH	SO_4^{2-} ion (μg.) Added	Recovered
Methyl sulphate	40	5·5	20	20
Ethyl sulphate	40	5·5	20	20
Ethanolamine sulphate	40	5·5	15	16
n-Propyl sulphate	40	5·5	20	20
*iso*Propyl sulphate	40	5·5	20	20
*iso*Propanolamine *O*-sulphate	40	5·5	20	20
n-Butyl sulphate	40	5·5	20	20
sec.-Butyl sulphate	40	5·5	20	20
Propane-1:3-diol disulphate	20	5·1	15	16
n-Pentyl sulphate	40	4·5	20	19
n-Hexyl sulphate	40	5·5	20	20
*cyclo*Pentyl sulphate	40	5·5	20	20
*cyclo*Hexyl sulphate	40	5·1	25	26
Tetrahydrofuran 2-methyl sulphate	40	5·5	20	20
Tetrahydropyran 2-methyl sulphate	40	5·5	20	19
Serine *O*-sulphate	20	5·7	20	20
	20	8·5	20	20
Threonine *O*-sulphate	20	5·7	20	20
	20	8·5	20	20
Hydroxyproline *O*-sulphate	20	5·7	10	11
	20	8·5	10	11
Holothurin	20	5·5	20	*
Dehydro*iso*androsterone sulphate†	6	5·1	25	28
Cortisone 21-sulphate†	20	5·6	15	24
2-(2:4-Dichlorophenoxy)ethyl sulphate†	40	5·1	25	30

* Too cloudy to measure. † Sodium salt.

Sodium cortisone 21-sulphate. Table 5 show the effect of increasing concentrations of this ester on recoveries of SO_4^{2-} ions in the presence of 0·5M-tris–acetic acid buffer. Similar results were obtained in the presence of 0·25M-tris–acetic acid and 0·25M-sodium acetate–acetic acid buffers. The results can be explained by assuming that the minimum concentration of SO_4^{2-} ions which must be present before $BaSO_4$ is precipitated quantitatively is comparatively high when cortisone 21-sulphate is present in high concentrations. At lower concentrations of the ester there are sufficient SO_4^{2-} ions present in the test solutions to cause complete precipitation for $BaSO_4$, but the SO_4^{2-} ion content of the controls is insufficient for this purpose. Support for this came from experiments in which extra SO_4^{2-} ion was added to both test and control solutions. This extra SO_4^{2-} ion was incorporated (as K_2SO_4) in the trichloroacetic acid solutions at a concentration of 12 μg. of SO_4^{2-} ion/ml. of trichloroacetic acid. Provided that the initial concentration of cortisone 21-sulphate did not exceed 0·01M, satisfactory recoveries of SO_4^{2-} ion could be obtained (Table 5).

Table 5. *Effect of various concentrations of sodium cortisone 21-sulphate on recoveries of potassium sulphate from solutions buffered at* pH 5·1 *with* 0·5M-*tris–acetic acid*

Concn. of cortisone 21-sulphate (mM)	SO_4^{2-} ion (μg.) Added	Recovered
80	20	3
80	10	2
40	10	3
20	10	14
20	15	24
10	10	14
5	15	18
2·5	15	17
10*	25	25
10*	15	15
10*	10	10
10*	5	5

* Extra SO_4^{2-} ion added to tests and blanks at a concentration of 12 μg. of SO_4^{2-} ion/ml. of trichloroacetic acid.

Table 6. *Effect of various compounds on the recoveries of potassium sulphate from aqueous solution*

Compound	Concn. (M)	SO_4^{2-} ion (μg.) Added	Recovered
Glucose	0·25	20	20
NaCl	0·10	20	20
$BaCl_2$	0·10	20	7
$MgCl_2$	0·10	20	20
Na_2SO_3	0·10	20	*
$MnCl_2$	0·10	20	20
$ZnCl_2$	0·10	20	20
NaF	0·10	20	20
KCN	0·10	20	20
$Na_4P_2O_7$	0·10	20	15
	0·02	20	19
	0·01	20	20
Hydroxylamine–HCl	0·10	20	20
Semicarbazide–HCl	0·10	20	20
Ethylenediaminetetra-acetic acid	0·10	20	20

* Too cloudy to measure.

Dehydroisoandrosterone sulphate and Crag herbicide. An initial concentration of 3 mM-DHAS in 0·5M-tris–acetic acid buffer resulted in slightly high recoveries of SO_4^{2-} ion (approx. 10%). Recoveries were good when extra SO_4^{2-} ion was added with the trichloroacetic acid to test and control solutions at a concentration of 10 μg. of SO_4^{2-} ion/ml. of trichloroacetic acid. In the presence of 0·5M-sodium acetate–acetic acid buffer, recoveries were good without the need to add extra SO_4^{2-} ion. Recoveries of SO_4^{2-} ion from buffered solutions of Crag herbicide also tended to be high. However, good recoveries were obtained when extra SO_4^{2-} ion was added as before, provided that the concentration of the ester did not exceed 0·01M.

Some common enzyme inhibitors and activators. Method B was used for these experiments. Recoveries of SO_4^{2-} ion from unbuffered solutions containing $BaCl_2$ or $Na_4P_2O_7$ (at concentrations greater than 0·01M) were low, and solutions containing Na_2SO_3 were too cloudy to read (Table 6).

Recoveries of sulphate from preparations of glycosulphatase

Several methods for the estimation of enzymically liberated SO_4^{2-} ion have been used in the study of glycosulphatase (see Dodgson & Spencer, 1957). All these methods suffer from limitations of one sort or another but the benzidine micromethod developed by Dodgson & Spencer (1953) can probably be regarded as the most sensitive and generally applicable of these methods. However, the method is laborious and time-consuming and demands a high degree of manipulative skill. It also suffers from the disadvantage of being inapplicable in the presence of concentrations of potassium glucose 6-*O*-sulphate (the substrate for glycosulphatase) greater than 0·02M. Attempts were therefore made to use the present turbidimetric method for the assay of glycosulphatase. Although the sensitivity of this method compares unfavourably with that of the benzidine procedure, this disadvantage would be outweighed by the simplicity and rapidity of the method and its applicability over a wide range of experimental conditions.

Crude extracts of the visceral regions of *Littorina littorea* (one of the best sources of glycosulphatase) gave cloudy suspensions when mixed with trichloroacetic acid. These suspensions could not be centrifuged under routine centrifuging conditions. However, with partially purified preparations of the enzyme (Dodgson, 1961) no cloud or precipitate appeared on addition of trichloroacetic acid. Seven separate preparations of the enzyme behaved similarly. The following procedure was used to check recoveries of SO_4^{2-} ion from these preparations. The enzyme solution (0·05 ml.) was incubated at 38° with 0·05 ml. of a solution of K_2SO_4 in water, sodium acetate or tris. After 1 hr., 1·4 ml. of 4% trichloroacetic acid was added and thereafter the procedure was exactly as described for method B. Control determinations were made in which water or buffer was substituted for the K_2SO_4 solution. Test solutions were measured against controls. Good recoveries were obtained in all cases (Table 7).

Good recoveries of SO_4^{2-} ion were also obtained from diluted preparations of the digestive juice of the snail

(*Helix pomatia*) (see Dodgson, 1961) and from a purified arylsulphatase preparation obtained from snail digestive gland. The method was subsequently used to study the hydrolysis of sodium cortisone 21-sulphate by these preparations.

Recoveries of sulphate from rat-tissue homogenates

In these experiments 0·2 ml. of a 4% (w/v) suspension of the tissue in M-tris–acetic acid buffer, pH 6·5, was incubated for 1 hr. with an equal volume of an aqueous solution of K_2SO_4. Trichloroacetic acid (4·6 ml. of 4%) was added and, after standing for 60 min. at −5°, precipitated protein was centrifuged down. A portion (4 ml.) of the clear supernatant was added to 1 ml. of the $BaCl_2$–gelatin reagent and thereafter method A was followed. Appropriate control determinations were made. Recoveries of SO_4^{2-} ion from liver preparations were poor (Table 8) unless extra SO_4^{2-} ion was added with the trichloroacetic acid (13 μg. of SO_4^{2-} ion/ml. of trichloroacetic acid).

Analysis of ester sulphates

The turbidimetric method was readily adapted to the analysis of ester sulphates. The ester sulphate (usually about 1 mg.) was dissolved in 1 ml. of water. A portion (0·02 ml.)

Table 7. *Recoveries of potassium sulphate in the presence of enzyme preparations*

Reaction mixtures were incubated for 1 hr. at 37·5° before analysis.

Enzyme preparation	Incubation medium	Concn. (M)	SO_4^{2-} ion (μg.) Added	Recovered
Littorina littorea concentrate	Water	—	30	30
			25	26
			20	20
			15	16
			10	10
	Sodium acetate	0·5	30	29
			20	20
			10	9
	Tris	0·25	30	29
			20	20
			10	10
		0·5	20	20
Snail digestive juice (diluted 10-fold)	Tris	0·5	20	19
			10	10
Snail digestive juice (diluted 30-fold)	Tris	0·5	10	10
Snail digestive-gland arylsulphatase	Tris	0·5	10	10

Table 8. *Recoveries of potassium sulphate from various rat-tissue suspensions*

Tissue suspensions (at a final concentration of 2%, w/v) were incubated with K_2SO_4 in the presence of 0·5 M-tris–acetic acid, pH 6·5, for 1 hr. at 37·5° before determination of SO_4^{2-} ion.

Tissue	Departure from normal reaction mixture	SO_4^{2-} ion (μg.) Added	Recovered
Liver	None	11	5
		31	15
		62	69
		99	116
		124	141
	40 μg. of SO_4^{2-} ion present in trichloroacetic acid	11	*
		31	29
		62	60
		99	94
	60 μg. of SO_4^{2-} ion present in trichloroacetic acid	11	10
Spleen	60 μg. of SO_4^{2-} ion present in trichloroacetic acid	11	14
Lung	60 μg. of SO_4^{2-} ion present in trichloroacetic acid	11	12
Heart	60 μg. of SO_4^{2-} ion present in trichloroacetic acid	11	12

* Results were inconsistent.

Table 9. *Analytical results for the* SO_4^{2-} *ion content of various sulphate esters*

Sulphate ester	SO_4^{2-} ion content (%) Found	Theory
Potassium *n*-amyl sulphate	46·0	46·6
Potassium *n*-butyl sulphate	50·3	49·2
Potassium *sec.*-butyl sulphate	50·3	52·3
Potassium *iso*propyl sulphate	53·9	53·9
Potassium tetrahydropyran 2-methyl sulphate	43·7	41·2
Potassium tetrahydrofuran 2-methyl sulphate	46·0	43·2
Dipotassium propane-1:2-diol disulphate	59·5	61·5
Potassium serine *O*-sulphate monohydrate	40·2	40·0
Potassium threonine *O*-sulphate monohydrate	38·2	37·6
Potassium hydroxyproline *O*-sulphate	39·4	38·5
Potassium *p*-nitrophenyl sulphate	36·3	37·4
Potassium *m*-cresyl sulphate	41·3	42·5
Dipotassium 2-hydroxy-5-nitrophenyl sulphate dihydrate	27·7	27·7
Potassium glucose 6-*O*-sulphate	32·8	32·3
Potassium methyl sulphate	65·0	64·1
Sodium dehydro*iso*androsterone sulphate	33·6	24·6
Sodium dehydro*iso*androsterone sulphate	26·2*	24·6

* After intermediate centrifuging (see text).

was transferred to a 100 mm. × 6 mm. stout-walled Pyrex tube containing 0·3 ml. of 4% trichloroacetic acid. The tube was sealed in an oxygen–coal gas flame and placed in an oven at 110°. Heating was continued for at least 4 hr., although it was generally convenient to leave the tubes overnight. After cooling, the tubes were shaken and allowed to stand for 5 min. in an upright position before opening. A portion (0·3 ml.) was withdrawn with a tapered constriction pipette and added to 0·1 ml. of the $BaCl_2$–gelatin reagent contained in a 50 mm. × 6 mm. test tube. After mixing, the extinction was measured in 1 cm. microcells against a reagent blank. Some sulphate esters, on hydrolysis, yielded parent compounds which absorbed in the 360 mμ region and in these cases spectrophotometric measurements were made at 500 mμ. In one case (DHAS) the trichloroacetic acid solution, after heating, was cloudy and the final SO_4^{2-} ion figure was high (Table 9). Good results were obtained by centrifuging the cloudy solution before removing the 0·3 ml. sample.

DISCUSSION

The difficulties associated with the measurement of microgram quantities of SO_4^{2-} ion under a wide variety of experimental conditions have long been recognized (see Dodgson & Spencer, 1953, 1957; Lloyd, 1959; Cope, 1931). In addition many of the methods which are available for the determination of SO_4^{2-} ion are time-consuming and exacting or, when used for studies on the enzymic and non-enzymic hydrolysis of sulphate esters, are very wasteful of starting material. The adaptations of the turbidimetric barium sulphate method which have been described above have the advantage of simplicity and rapidity and, on the whole, are less subject to interference from other materials which may be present. Interference is probably minimized by the considerable dilution which occurs when the relatively large volume of trichloroacetic acid is added to the reaction mixture.

The method is less sensitive than either the benzidine or the chloranilate procedure, but this fact is compensated for by the simplicity of the method. Enzyme experiments with purified glycosulphatase, which normally take 1 or 2 days to complete, can be accomplished in 2 or 3 hr. Moreover, it is a simple matter to check recoveries of SO_4^{2-} ion whenever new experimental conditions are to be used.

One drawback to the method, when it is used for enzyme studies, is that care must be taken to ensure that sufficient substrate is present to enable kinetic interpretations of results to be made. For example, with method B, complete hydrolysis of 0·05 ml. of 0·02 M-potassium glucose 6-*O*-sulphate by an equal volume of a glycosulphatase solution would yield a total of 96 μg. of SO_4^{2-} ion and hydrolysis of 10% of the substrate would yield a sufficient amount of SO_4^{2-} ion (9·6 μg.) to be measured accurately. On the other hand, 10% hydrolysis of 0·05 ml. of a 2 mM-solution of the substrate would yield only 0·96 μg. of SO_4^{2-} ion. Fortunately, the optimum substrate concentration for glycosulphatase activity is high (0·04 M-potassium glucose 6-*O*-sulphate; see Dodgson, 1961) and the method has been of great value in studies on this enzyme. On the other hand, the method is not entirely suitable for the assay of the 3β-steroid sulphatase, which is also present in glycosulphatase preparations, since the optimum substrate concentration of this enzyme is less than 1 mM. The activity of this enzyme must therefore be measured at substrate concentrations greater than optimum when some inhibition by excess of

substrate is obtained (see Dodgson, 1961). The method would also appear to be of doubtful value for use with crude mammalian-tissue preparations in view of the need to add relatively large amounts of extra SO_4^{2-} ion to tests and controls before good recoveries of SO_4^{2-} ion can be obtained.

The method has also been successfully used to follow the alkaline- and acid-hydrolysis rates of the *O*-sulphates of serine and threonine (K.S. Dodgson, A. G. Lloyd & N. Tudball, to be published) and to measure the ester sulphate content of chondroitin sulphate preparations after hydrolysis with hydrochloric acid (R. G. Price, unpublished results).

SUMMARY

1. Three variations of a turbidimetric method for the determination of small amounts of inorganic sulphate have been described.

2. One variation, covering the range 0–200 μg. of SO_4^{2-} ion, is suitable for following the non-enzymic hydrolysis of monosaccharide sulphates by hydrazine; a second variation, covering the range 0–40 μg. of SO_4^{2-} ion, has been developed for the assay of purified glycosulphatase preparations; the other variation, covering the range 0–12 μg. of SO_4^{2-} ion, has been successfully used for the microanalysis of various ester sulphates.

3. The method can be used in the presence of hydrazine, imidazole, various buffers, anions, cations, monosaccharide mono- and di-sulphate and other sulphate esters. SO_3^{2-}, Ba^{2+} and $P_2O_7^{4-}$ ions (in concentrations greater than 0·01 M), and a large number of polysaccharide sulphate esters which were tested, interfered with the method. Other limitations of the method are described.

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Potassium Glucose 6-*O*-Sulphate as a Substrate for Glycosulphatase

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During a study of the glycosulphatase of *Littorina littorea*, Dodgson & Spencer (1954) observed anomalies in the behaviour of the enzyme towards increasing concentrations of potassium glucose monosulphate (prepared by direct sulphation of glucose and presumed to be glucose 6-*O*-sulphate), which they suggested might be due to the heterogeneity of the substrate preparation. Evidence for the presence of more than one sulphate ester in the preparation was obtained by paper chromatography and from experiments in which it was shown that about 25% of the material could be desulphated by treatment with 0·33 M-hydrazine at pH 5·3 and 38° (see also Egami, 1938, 1940, 1942). Further work on the enzyme was deferred until pure potassium glucose 6-*O*-sulphate could be obtained.

Recent work from these laboratories (Lloyd, 1960) has shown that glucose, potassium glucose 6-*O*-sulphate and dipotassium glucose disulphate are present in preparations of potassium glucose monosulphate obtained by direct sulphation of glucose. The present study shows that the partial hydrolysis of such preparations by hydrazine can be attributed to the hydrolysis of one of the sulphate groups present in potassium glucose

disulphate. It has also been demonstrated that heterogeneity of substrate is not responsible for the anomalous behaviour of *Littorina* glycosulphatase. Attempts to detect carbohydrate sulphatases in mammalian tissues are also described.

MATERIALS AND METHODS

Carbohydrate sulphates. Potassium glucose 6-*O*-sulphate, dipotassium glucose disulphate and the potassium salts of *N*-acetylglucosamine sulphate and *N*-acetylgalactosamine 6-*O*-sulphate were prepared by the method of Lloyd (1960). Potassium glucose 3-*O*-sulphate was obtained from di*iso*propylidene glucose as described by Dodgson & Spencer (1954). Potassium galactose 6-*O*-sulphate was prepared by definitive synthesis (Lloyd, 1960), by column electrophoresis of the products of the direct sulphation of galactose at 37° (Lloyd, 1960) and by a similar method after direct sulphation at 60–70° (cf. Turvey & Clancy, 1959). Dipotassium galactose disulphate was separated by column electrophoresis from the products of the direct sulphation of galactose at 60–70° (cf. Lloyd, 1960). Potassium chondroitin sulphate A and C were prepared from cartilage chondroitin sulphate (Dodgson, Lloyd & Spencer, 1957) by the fractionation procedure of Meyer, Davidson, Linker & Hoffman (1956). Potassium *N*-acetylchondrosine sulphate was prepared by enzymic degradation of chondroitin sulphate A according to Dodgson & Lloyd (1958), and a crude mixture of the corresponding higher oligosaccharides (tetra-, hexa- and octa-saccharides) was obtained by degrading chondroitin sulphate A with testicular hyaluronidase (cf. Hoffman, Meyer & Linker, 1956).

Enzyme preparations. A partially purified preparation of glycosulphatase was prepared from the visceral region of *Littorina* according to the procedure of Dodgson (1961*b*). Homogenates of mammalian tissues were prepared as described by Dodgson, Spencer & Thomas (1953).

Determination of enzyme activity. The activity of the *Littorina* preparation was followed by determining liberated SO_4^{2-} ions by the method of Dodgson (1961*a*; method B). In following the activity of other enzyme preparations the benzidine micromethod (Dodgson & Spencer 1953) or the chloranilate procedure of Lloyd (1959) was used. An incubation temperature of 38° was used in all enzyme experiments.

Determination of rates of hydrolysis of carbohydrate sulphates by hydrazine. The non-enzymic hydrolysis of the mono- and di-sulphate esters of glucose and galactose was followed by estimating liberated SO_4^{2-} ions by the method of Dodgson (1961*a*). The following general procedure was adopted. A portion (1 ml.) of a solution (0·05 M) of the sulphate ester in 0·5 M-sodium acetate–acetic acid buffer, pH 5·3, was incubated at 38° with 1 ml. of a 0·66 M-hydrazine hydrate solution which was prepared by adjusting the requisite amount of the base to pH 5·3 with 0·5 M-acetic acid and diluting to the appropriate volume with 0·5 M-sodium acetate–acetic acid buffer, pH 5·3. At suitable time intervals, 0·2 ml. samples were withdrawn and added to 3·8 ml. of 4% trichloroacetic acid. Thereafter the method was as described by Dodgson (1961*a*; method A). Control determinations were made in which substrate and hydrazine were incubated separately, 0·1 ml. samples of each being withdrawn at the appropriate time and added to 3·8 ml. of 4% trichloroacetic acid. When the amount of SO_4^{2-} ion liberated in the test solutions was greater than that covered by the calibration curve, then smaller samples (0·1 or 0·05 ml.) were withdrawn and, after mixing with the trichloroacetic acid, the necessary volume of water (0·1 or 0·15 ml.) was added before proceeding.

The effect of hydrazine on chondroitin sulphates A and C was examined as follows. A portion (2 ml.) of a 2% (w/v) solution of the sulphate ester in 0·5 M-barium acetate–acetic acid buffer, pH 5·3, was incubated for 48 hr. at 38° with an equal volume of a 0·66 M-hydrazine hydrate solution prepared as described above. The incubation mixture was acidified with a drop of conc. HCl and the precipitated $BaSO_4$ determined gravimetrically.

RESULTS

Hydrolysis of carbohydrate sulphates by hydrazine

Sulphate esters of glucose. Fig. 1 shows the effects of hydrazine on the potassium salts of the 3-*O*-, 6-*O*- and di-sulphates of glucose. The 3-*O*- and 6-*O*-sulphates were hydrolysed only slowly by hydrazine at 38°, whereas the corresponding disulphate was relatively rapidly hydrolysed under the same conditions. The amount of SO_4^{2-} ion liberated after 3 days was equivalent to approx. 31% of the total available in the substrate. Addition of extra hydrazine at this point does not significantly affect the rate of hydrolysis. The shape of the curve suggests that one sulphate group only is readily hydrolysed by hydrazine. Separate experiments in which the 3-*O*-, 6-*O*- and disulphates were incubated with hydrazine in the

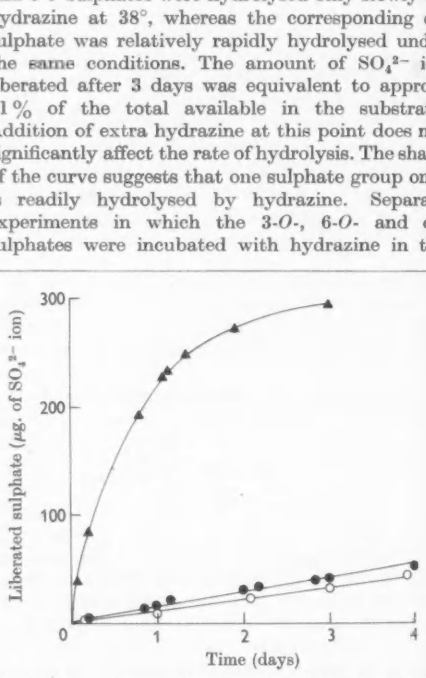

Fig. 1. Curves showing the rates of hydrolysis of 25 mM-solutions of potassium glucose 6-*O*-sulphate, potassium glucose 3-*O*-sulphate and dipotassium glucose disulphate by 0·33 M-hydrazine at 38° in the presence of 0·5 M-sodium acetate–acetic acid buffer, pH 5·3. Experimental points are calculated for a volume of 0·2 ml. of incubation mixture. ○, Potassium glucose 3-*O*-sulphate; ●, potassium glucose 6-*O*-sulphate; ▲, dipotassium glucose disulphate.

presence of glucose (final concn. 0·1 M) showed that glucose did not significantly affect the rates of hydrolysis of the three substrates.

Sulphate esters of galactose. Dipotassium galactose disulphate was relatively rapidly hydrolysed by hydrazine (Fig. 2), the amount of SO_4^{2-} ion liberated after 3 days being equivalent to approx. 41% of the total available in the substrate. Addition of extra hydrazine at this point does not significantly increase the rate of hydrolysis. The shape of the curve suggests that one sulphate group only is readily hydrolysed. The two different curves shown for galactose 6-*O*-sulphate are of some interest. The lower curve was obtained with substrate prepared by definitive synthesis and with substrate obtained by sulphation of galactose at 37°. The upper curve was obtained with substrate prepared by sulphation of galactose at 60–70°. No disulphate could be detected in this preparation and the curve may possibly reflect the presence of small amounts of an isomeric monosulphate in the preparation. Rees (1960 and personal communication) has indicated that crude glucose monosulphate and galactose monosulphate preparations obtained by direct esterification at high temperatures contain, in addition to the 6-*O*-sulphate and disulphate, small amounts of isomeric monosulphates.

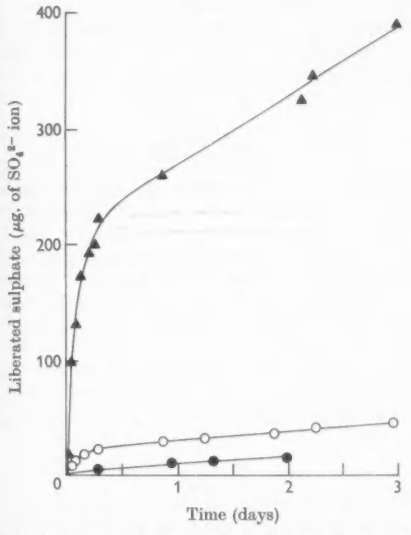

Fig. 2. Curves showing the rates of hydrolysis of 25 mM-solutions of potassium galactose 6-*O*-sulphate and dipotassium galactose disulphate by 0·33 M-hydrazine at 38° in the presence of 0·5 M-sodium acetate–acetic acid buffer, pH 5·3. Experimental points are calculated for a volume of 0·2 ml. of incubation mixture. ●, Potassium galactose 6-*O*-sulphate prepared by direct sulphation at 37° and by definitive synthesis (see text); ○, potassium galactose 6-*O*-sulphate prepared by direct sulphation at 60–70°; ▲, dipotassium galactose disulphate.

Effect of pH. According to Egami (1938) the liberation of SO_4^{2-} ion from crude preparations of glucose monosulphate proceeded optimally at pH 5·2 and this led him to suggest that hydrazine could be regarded as a model for the enzyme glycosulphatase, a view which he subsequently modified. The effect of pH on the hydrolysis of the disulphates of glucose and galactose by hydrazine over a 90-min. period at 38° is shown in Fig. 3. Between the values 4·8 and 6·9, pH had relatively little effect on the rate of hydrolysis of either compound. Below pH 4·8 the rate of hydrolysis increased significantly. The curves were remarkably similar although galactose disulphate was desulphated at approximately four times the rate of that for the corresponding glucose derivative over this particular incubation period. These results differ from those obtained by Egami (1938) with crude glucose monosulphate preparations, although a strict comparison is not valid since Egami had to use long incubation periods and other experimental conditions were not identical.

Chondroitin sulphates A and C. Hydrazine was without effect on either compound over a period of 48 hr.

Effect of imidazole on carbohydrate sulphates

Imidazole is capable of hydrolysing various esters (see Bruice & Schmir, 1956, 1957), including some sulphate esters (K. S. Dodgson, unpublished results), and there is evidence that the active

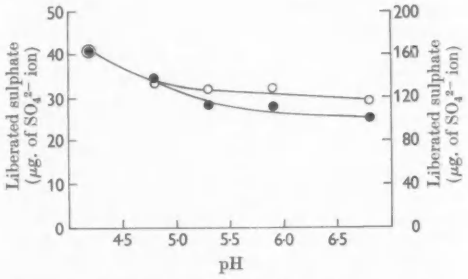

Fig. 3. Effect of pH on the hydrolysis of 25 mM-solutions of dipotassium glucose disulphate and dipotassium galactose disulphate by 0·33 M-hydrazine in the presence of 0·5 M-sodium acetate–acetic acid buffer. Incubation was for 90 min., at 38°, and experimental points are calculated for a volume of 0·2 ml. of incubation mixture. ○, Dipotassium glucose disulphate (left-hand ordinate scale); ●, dipotassium galactose disulphate (right-hand ordinate scale).

centres of several hydrolytic enzymes are functionally dependent upon imidazole groupings. It was therefore of interest to check the possibility that imidazole could also catalyse the hydrolysis of carbohydrate sulphate esters in a manner similar to that of hydrazine. The esters (0·1 ml. of a 0·05 M-solution in 0·5 M-sodium acetate–acetic acid buffer) was incubated with 0·1 ml. of a 0·4 M-solution of imidazole in the same buffer over a period of 18 hr. at 38°. Three different conditions of pH were used (pH 5·0, 6·5 and 7·5) with each substrate. Liberated SO_4^{2-} ion was determined according to Dodgson (1961*a*; method A). The 3-*O*-, 6-*O*- and di-sulphates of glucose and the 6-*O*-sulphate and disulphate of galactose were not affected by imidazole, irrespective of the pH of the incubation mixture.

Anomalous substrate concentration–activity curve of glycosulphatase

The substrate concentration–activity curve obtained by Dodgson & Spencer (1954) during the original studies on the enzyme is reproduced in part (as the continuous line) in Fig. 4. Also reproduced in this Figure are the experimental points for a more highly purified preparation of the enzyme when pure potassium glucose 6-*O*-sulphate was used under the same conditions of buffer and pH. The experimental points fit closely to the original curve. Also shown are the curves obtained when pure samples of potassium glucose 6-*O*-sulphate and dipotassium glucose disulphate were mixed together in the proportions 3:1 and 4:1 and the mixtures then used as assay substrates. Again the experimental points fit reasonably well on the original curve. Clearly the anomalous nature of the original curve cannot be attributed to the presence of more than one sulphate ester in the preparations of crude glucose monosulphate as originally suggested by Dodgson & Spencer (1954). It will be shown later (Dodgson, 1961*b*) that no anomalies are present in substrate concentration–activity curves which are determined in the presence of 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–acetic acid buffer.

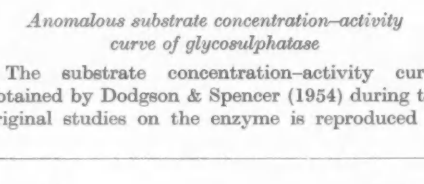

Fig. 4. Experimental points showing the effect of substrate concentration on the activity of partially purified preparations of *Littorina* glycosulphatase acting, in the presence of 0·5 M-sodium acetate–acetic acid buffer, pH 5·8, on the following substrates: ○ and ×, two separate preparations of potassium glucose 6-*O*-sulphate; ▲, potassium glucose 6-*O*-sulphate and dipotassium glucose disulphate mixture (4:1, w/w); ●, potassium glucose 6-*O*-sulphate and dipotassium glucose disulphate mixture (3:1, w/w). Where mixed substrates were used, calculations of molarity of substrate were made as though the 6-*O*-sulphate only was present. The continuous line is a reproduction, on the same scale, of the curve originally obtained by Dodgson & Spencer (1954) with crude preparations of potassium glucose monosulphate.

The optimum pH of the *Littorina* enzyme when acting on 0·04 M-potassium glucose 6-*O*-sulphate in the presence of 0·5 M-sodium acetate–acetic acid buffer was in the region of 5·8 (Fig. 5). This agrees closely with the value of 5·85 obtained by Dodgson & Spencer (1954) with crude preparations of potassium glucose monosulphate.

Examination of mammalian tissues for carbohydrate sulphatases

Previous attempts to detect enzymes capable of liberating SO_4^{2-} ion from mono- or poly-saccharide sulphates have resulted in conflicting findings. Thus K. S. Dodgson & B. Spencer (unpublished results) failed to detect glycosulphatase in rat liver whereas T. Soda (personal communication) claimed

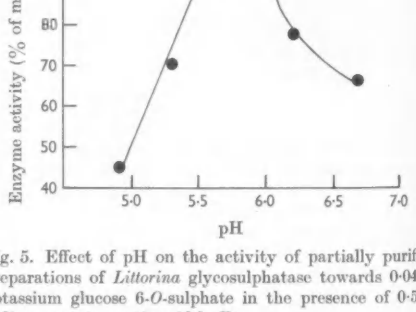

Fig. 5. Effect of pH on the activity of partially purified preparations of *Littorina* glycosulphatase towards 0·04 M-potassium glucose 6-*O*-sulphate in the presence of 0·5 M-sodium acetate–acetic acid buffer.

that the enzyme was present in whale and rabbit livers. Neuberg & Cahill (1936) and Dohlman & Friedenwald (1955) failed to observe liberation of sulphate from chondroitin sulphate by various tissue preparations. In contrast, the work of Dziewiatkowski (1956) and Dohlman (1956) suggested that enzymic liberation of sulphate from this compound did occur *in vivo*, and the results of Hall & Gardiner (1955) indicated that enzymic desulphation of endogenous polysaccharide sulphate occurred in pig-pancreas preparations.

Two types of substrate were used during the present attempts to detect mammalian carbohydrate sulphatases, namely simple monosaccharide sulphates (substrates for glycosulphatase) and chondroitin sulphate and the sulphated oligosaccharides derived therefrom (substrates for chondrosulphatase). The following tissues were examined: (*a*) fresh and acetone-dried pancreas, lung, kidney, liver, spleen, testis, ovary, brain and costal cartilage from M.R.C. hooded rats, age 3 and 12 months; (*b*) fresh and acetone-dried heart, knee cartilage, costal cartilage, pancreas, lung, kidney, liver, spleen, testis, ovary and brain of 4-day-old pigs; (*c*) acetone-dried post-mortem samples of pancreas, lung, kidney, liver, spleen and brain from a human male, age 58 years. All tissues were examined as 2% (w/v) suspensions in 0·2M-sodium acetate–acetic acid, tris–acetic acid and glycine–HCl buffers at several pH values within the range 3·5–8·5.

The potassium salts of the 6-*O*-sulphates of glucose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine were used as substrates at a concentration of 25 mM. Potassium chondroitin sulphate A and the unfractionated mixture of higher oligosaccharides obtained by degradation of chondroitin sulphate with testicular hyaluronidase were used at a concentration of 0·5%, whereas the concentration of *N*-acetylchondrosine sulphate was 0·05%. Incubation of enzyme preparation and substrate was for 4, 12, 24 and 48 hr. at 38°.

In no case was it possible to detect liberation of SO_4^{2-} ion by the various tissue preparations.

DISCUSSION

It is now clear that the liberation of SO_4^{2-} ion from preparations of crude glucose monosulphate by hydrazine reflects the presence of glucose disulphate in such preparations. It has further been established that the anomalous substrate concentration–activity obtained when crude glucose monosulphate preparations were used as a substrate for *Littorina* glycosulphatase (Dodgson & Spencer, 1954) cannot be attributed to the presence in the preparation of sulphate esters other than the 6-*O*-sulphate.

The behaviour of hydrazine towards carbohydrate sulphate esters has been studied at some length by Egami (1938, 1940, 1942), who concluded that one of the prerequisites for the reaction was the presence, in the ester, of a potential reducing group, although hydrazine could be recovered unchanged at the end of the reaction. On these grounds chondroitin sulphates A and C would not be expected to undergo desulphation by hydrazine. The structure of glucose disulphate and galactose disulphate is clearly of some interest since in each case one of the two sulphate groups appears to be particularly labile to hydrazine. It is probably reasonable, in view of the high yields of the 6-*O*-sulphates by direct sulphation of glucose or galactose, to assume that one of the sulphate groups in the corresponding disulphates occupies position 6 of the hexose residue. The finding that potassium glucose 3-*O*-sulphate is desulphated at about the same rate as the corresponding 6-*O*-sulphate may further suggest that position 3 in the disulphate is not esterified. The principal difference between glucose and galactose lies in the relative spatial distribution of the hydroxyl group at C-4. It may therefore be of some significance that galactose disulphate is desulphated at about four times the rate of that for glucose disulphate over short incubation periods. However, examination of the infrared spectrum of galactose disulphate (Lloyd & Dodgson, 1961) has not provided any evidence for the presence of an ester sulphate grouping at C-4. Further progress now depends on the preparation of hexose monosulphates of known constitution.

Attempts to detect carbohydrate sulphatases in mammalian tissues have been unsuccessful, and it seems clear that such enzymes are confined to relatively simple organisms. Dodgson & Lloyd (1957) recently established that the chondrosulphatase of *Proteus vulgaris* was without action on polymer chondroitin sulphate. The enzyme was associated with an aminopolysaccharase which is able to depolymerize chondroitin sulphate. The sulphated oligosaccharides resulting from depolymerization were readily desulphated by chondrosulphatase. Similar conclusions have since been made for the enzyme desulphation of heparin by enzyme preparations from *Flavobacterium heparinum* (Meyer, Linker, Hoffman & Korn, 1957). For these reasons, sulphated oligosaccharides were tested as substrates during the present work. However, no enzyme capable of desulphating these substrates could be detected.

Potassium glucose 6-*O*-sulphate, prepared by the method of Lloyd (1960), is clearly a suitable substrate for the assay of glycosulphatase. Further support for this conclusion is presented in the next paper (Dodgson, 1961*b*).

SUMMARY

1. Solutions of hydrazine catalyse the partial desulphation of the disulphate esters of glucose and galactose. Quantitative results show that, under standard conditions (pH 5·3 in 0·5 M-sodium acetate–acetic acid buffer at 38°), galactose disulphate is desulphated more rapidly than the corresponding glucose derivative and suggest that in each case one ester sulphate group only is readily removed.

2. The 6-*O*-monosulphate esters of glucose and galactose and the 3-*O*-monosulphate of glucose are not readily desulphated by hydrazine.

3. Unsuccessful attempts have been made to detect the presence in mammalian tissues of enzymes capable of liberating sulphate from monosaccharide sulphate esters, chondroitin sulphates A and C and from various sulphated oligosaccharides derived from chondroitin sulphate A.

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Glycosulphatase: Observations on the Activity of Partially Purified Preparations towards the Sulphate Esters of Certain Monosaccharides and Steroids

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The existence of glycosulphatase in the digestive organs of certain tropical marine molluscs was first noted by Soda & Hattori (1931). Between 1931 and 1948, in a series of publications (see Dodgson & Spencer, 1957*a*), Soda and his coworkers described various properties of the enzyme, although much of the quantitative work must be regarded with caution because of the impure nature of the enzyme substrate (potassium glucose monosulphate, see Dodgson & Lloyd, 1961). More recently Dodgson & Spencer (1954) showed the enzyme to be present in extracts of the digestive organs of some marine molluscs from British waters, including the common limpet (*Patella vulgata*) and the large periwinkle (*Littorina littorea*). Subsequently Roy (1956), during a study of the 3β-steroid sulphatase of *Patella*, noted that sodium cortisone 21-sulphate was desulphated by his enzyme preparations (see also Savard, Bagnoli & Dorfman, 1954) and suggested that glycosulphatase was the enzyme responsible.

In the present paper the properties of partially purified preparations of *Littorina* glycosulphatase are described. Evidence is also presented which

suggests that the ability of these preparations to hydrolyse sodium cortisone 21-sulphate cannot be attributed to the presence of glycosulphatase.

MATERIALS AND METHODS

Enzyme substrates. References to the methods of preparation of various carbohydrate and other sulphate esters used as enzyme substrates during the course of the work have previously been given (Dodgson, 1961; Dodgson & Lloyd, 1961). Sodium dehydro*iso*androsterone sulphate and sodium cortisone 21-sulphate were kindly supplied by Dr A. B. Roy and Merck and Co. Inc., N.J., U.S.A., respectively. Phenolphthalein glucuronide was purchased from L. Light and Co. Ltd. and *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide was a gift from Dr P. G. Walker.

Molluscs. Supplies of *Littorina* were purchased from the local fish-market or were collected at low tide from the beach at Sully, Glamorgan. Snails (*Helix pomatia*) were obtained from L. Haig, Beambrook, Newdigate, Surrey, and were starved for 4 days before use.

Determination of the activity of various enzymes

An incubation temperature of 38° was used in all enzyme experiments.

Glycosulphatase. Glycosulphatase activity towards various monosaccharide monosulphate esters was followed by estimating enzymically liberated SO_4^{2-} ion. With crude enzyme extracts the SO_4^{2-} ion was measured by the benzidine ultramicro method of Spencer (1960) or the chloranilate procedure of Lloyd (1959). With the final *Littorina* enzyme preparation and with *Helix* preparations, the turbidimetric $BaSO_4$ procedure of Dodgson (1961) was used as described below. The buffered substrate (0·05 ml.) was incubated with an equal volume of the enzyme solution. After 1 hr., 1·4 ml. of 4% trichloroacetic acid was added and thereafter method B (see Dodgson, 1961) was followed. The concentration of enzyme was adjusted so that no more than 35 μg. of SO_4^{2-} ion was liberated and suitable control determinations were made in which enzyme and substrate were incubated separately and mixed only just before the addition of trichloroacetic acid. Test solutions were measured against controls.

Sulphatase activity towards dehydroisoandrosterone sulphate and cortisone 21-sulphate. Lloyd's (1959) chloranilate procedure or Spencer's (1960) benzidine ultramicro method was used with crude enzyme preparations. With the purified *Littorina* preparation or with the *Helix* enzyme, the turbidimetric $BaSO_4$ method was used as described in the previous section except that the 4% trichloroacetic acid contained 12 μg. of SO_4^{2-} ion/ml. (as K_2SO_4; see Dodgson, 1961). When it was necessary to use cortisone 21-sulphate at concentrations between 0·01 and 0·02 M, 0·025 ml. of substrate was incubated with an equal volume of enzyme and, after the addition of trichloroacetic acid, 0·05 ml. of water was added to make up the appropriate volume (see Dodgson, 1961).

Arylsulphatase. A micro-adaptation of the 4-nitrocatechol method of Dodgson & Spencer (1957*b*) was used. Dipotassium 2-hydroxy-5-nitrophenyl sulphate (0·02 ml. of a 0·01 M-solution in 0·5 M-sodium acetate–acetic acid buffer, pH 5·5) was incubated with an equal volume of enzyme for 1 hr. in a 50 mm. × 60 mm. test tube. Enzyme action was stopped and the liberated 4-nitrocatechol was converted into the red anionic form by addition of 0·2 ml. of a 0·4 M-solution of glycine which had been adjusted to pH 10 by adding solid NaOH. The extinction of the solution was measured at 515 mμ (ϵ_{515} 12 400) with the Hilger model H 700.308 spectrophotometer and the 1 cm. microcells and adaptor. Suitable control determinations were made in which enzyme and substrate were incubated separately and mixed only just before the addition of the glycine buffer. Enzyme concentration was adjusted so that spectrophotometric readings (log I_0/I) did not exceed 0·7.

β-Glucuronidase. The method used was a micro-adaptation of that described by Levvy (1952). Phenolphthalein glucuronide (0·02 ml. of a 2·5 mM-solution in 0·2 M-sodium acetate–acetic acid buffer, pH 4·0) was incubated for 1 hr. with an equal volume of enzyme solution. Thereafter the method followed that described in the previous section except that spectrophotometric readings were made at 554 mμ (ϵ_{554} 25 400). Suitable control determinations were made.

β-N-Acetylglucosaminidase. A micro-adaptation of the method of Findlay, Levvy & Marsh (1958) was used. *p*-Nitrophenyl *N*-acetyl-β-D-glucosaminide (0·01 ml. of a 0·01 M-solution in water) was incubated for 1 hr. with 0·02 ml. of enzyme solution and 0·01 ml. of 0·2 M-citric acid which had been adjusted to pH 4·4 by addition of 0·2 M-NaOH. Thereafter the method followed that described for arylsulphatase except that liberated *p*-nitrophenol was measured at 400 mμ (ϵ_{400} 18 200). The usual control determinations were made.

Chondrosulphatase. Ox-cartilage chondroitin sulphate A (final concn. 0·1% in 0·5 M-sodium acetate–acetic acid buffer, pH 5·0) was used as substrate and enzyme activity was followed by the method of Spencer (1960).

Chondroitinase. The method of Dodgson, Lloyd & Spencer (1957) was reduced in scale (to one-third). The assay substrate was that described in the preceding section.

Determination of nucleic acid. The ratio of the spectrophotometric readings at 280 and 260 mμ was used (Warburg & Christian, 1941).

EXPERIMENTAL AND RESULTS

Purification of Littorina *glycosulphatase*

Stage 1. The visceral organs of *Littorina* were dissected out and washed briefly in water before macerating (Townson and Mercer Ltd. macerator) for 2 min. with 5 vol. of acetone at 0°. After filtering at the pump the debris was re-macerated in fresh acetone as before, filtered and then exhaustively washed with cold acetone until the washings were colourless. The debris was dried *in vacuo* at 4° and subsequently stored at −10° until required.

Stage 2. The acetone-dried powder (10 g.) was suspended in 120 ml. of ice-cold water with the aid of a glass homogenizer. The pH of the suspension (usually approx. 9·0) was lowered to 7·0 with a few drops of acetic acid and the suspension was incubated at 38° for 15 min. Preliminary experiments showed that extraction of the acetone-dried powder with sodium acetate–acetic acid buffer at pH 7·0 was less efficient than extraction with water. After cooling to 2° the suspension was centrifuged at 6000 *g* (av.) and the resultant cloudy supernatant was held at 2° whilst the debris was washed by re-suspending it in 80 ml. of ice-cold

water and centrifuging as before. The supernatants were pooled and clarified by centrifuging at 78 000 *g* (av.) in the Spinco preparative ultracentrifuge. The clear extract was then dialysed in Visking dialysis bags (Hudes Merchandising Corp., 52 Gloucester Place, London, W. 1) for 24 hr. at 4° against several changes of water. The enzyme solution was transferred to new dialysis bags after 12 hr., otherwise a cellulase which was also present in the extract (cf. Roy, 1956) tended to rupture the dialysis tubing.

Stage 3. The dialysed extract was kept at 4° whilst the pH was lowered to 2·3 by the addition of 2N-HCl. After the extract had been standing for 2 min. the pH was quickly elevated to 4·6 with 2N-NaOH and, after standing for 2–3 hr., the whole was centrifuged at 0° and 6000 *g* (av.). This acid treatment (originally described by Dodgson & Spencer, 1953) destroyed most of the β-glucuronidase which is present in the extract and removed much other unwanted material. The cellulase was also apparently destroyed, since the extract was no longer capable of rupturing dialysis tubing. The clear supernatant was dialysed at 4° for 24 hr. against several changes of water.

Stage 4. The pH of the extract was adjusted to 6·7 and 0·4 ml. of a 5% solution of ribonucleic acid, which had been adjusted to the same pH, was added. A 2% solution of salmon-roe protamine sulphate at pH 6·7 was added until no further precipitation occurred. The precipitate was removed by centrifuging and a further 3 ml. of the protamine sulphate solution was added to the supernatant, which was then dialysed for 3 days at 4° against several changes of water. Precipitated material was removed by centrifuging and the pH of the clear supernatant elevated to 8·0. After standing for 2 hr. at 0° the excess of protamine sulphate which precipitated was removed by centrifuging and the clear supernatant was dialysed overnight at 4° against water. This stage resulted in an appreciable decrease in the nucleic acid content of the preparation.

Stage 5. Sufficient 2M-sodium acetate–acetic acid buffer, pH 5·7, was added to the extract to give a final concentration of 0·1M-acetate and the whole was cooled to 0°. Sufficient solid $(NH_4)_2SO_4$ was added (over a period of about 30 min., with gentle stirring) to give a final concentration equivalent to 55% saturation (calculated from the nomogram of Dixon, 1953, without correcting for temperature). After standing at 0° for 90 min. the precipitate was separated by centrifuging at 0° and discarded. The pH of the supernatant was lowered to 4·9 with acetic acid and sufficient $(NH_4)_2SO_4$ added at 0° to give a final concentration equivalent to 85% saturation (calculated as before). After standing for 2 hr. at 0° the precipitate was separated by centrifuging at 0° and 78 000 *g* (av.), dissolved in 10 ml. of water and dialysed for 24 hr. at 4° against several changes of water. The final enzyme solution was stored in plastic containers at −10°.

Table 1 shows the purification achieved for a preparation beginning with acetone-dried material prepared in the month of April and containing relatively low amounts of glycosulphatase. During the course of several preparations made in a period of about 18 months it has been noticed that glycosulphatase activity in *Littorina* varies considerably with season. Relatively small amounts of the enzyme can be detected in the winter months, whereas organisms collected in July have provided the most active preparation to date (approx. three times as active as the preparation described in Table 1). The purification procedure

Table 1. *Activities of some hydrolytic enzymes at various stages of the purification of extracts of the digestive organs of* Littorina littorea

The following arbitrary experimental conditions were used to measure enzyme activities during purification: glycosulphatase, 0·02M-glucose 6-*O*-sulphate in 0·5M-tris–acetic acid buffer, pH 5·5; cortisone 21-sulphatase, 5 mM-cortisone 21-sulphate in 0·25M-sodium acetate–acetic acid buffer, pH 5·6; dehydroisoandrosterone sulphate, 2 mM in 0·25M-sodium acetate–acetic acid buffer, pH 4·5. Conditions used for assay of the other enzymes are described in the text. Enzyme activities refer to an incubation period of 1 hr. and 1 unit is equal to 1 μg. of the particular product of hydrolysis which is eventually measured (see text). With chondroitinase, liberated reducing material has been arbitrarily expressed as glucose (see Dodgson, Lloyd & Spencer, 1957).

Purification stage	Yield (g. dry wt.)	Nucleic acid (%)	Glyco-sulphatase (units/g.)	Cortisone 21-sulphatase (units/g.)	3-β-Steroid sulphatase (units/g.)	β-*N*-Acetyl-glucos-aminidase (units/g.)	β-Gluc-uronidase (units/g.)	Aryl-sulphatase (units/g.)	Chondroitinase (μg. of glucose liberated/g.)	Chondro-sulphatase (units/g.)
Acetone-dried powder	10	—	3 200*	—	—	—	—	—	—	—
Stage 2	3·12	5	9 700	4 600	3 190	29 300	169 000	207 000	615	0
Stage 3	1·50	4	16 650	6 260	6 500	58 000	1 950	182 000	0	0
Stage 5	0·13	<1	70 100	50 300	26 700	5 240	2 615	712 000	0	0
			85 000†							

* Measured on a dialysed suspension of the powder. † Measured under optimum conditions.

results in the elimination of the chondroitinase and most of the β-glucuronidase and β-*N*-acetylglucosaminidase but other sulphatase enzymes are still present in appreciable amounts. Nucleic acid concentration has been considerably decreased and the preparation (which retains activity indefinitely when stored in the frozen state) is a suitable starting point for further purification studies.

Properties of the purified enzyme

Effect of substrate concentration and pH. Fig. 1 shows the effect of increasing concentrations of the 3-*O*-sulphate and 6-*O*-sulphate of glucose and the 6-*O*-sulphate of galactose on enzyme activity in the presence of 0·5 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)–acetic acid buffer at the appropriate optimum pH. The curve for glucose 6-*O*-sulphate no longer exhibits the anomalies observed by Dodgson & Spencer (1954) and Dodgson & Lloyd (1961) for sodium acetate–acetic acid-buffered solutions of the enzyme. The broken line in Fig. 1 shows the corresponding curve for the same concentration of enzyme acting in the presence of 0·5 M-sodium acetate–acetic acid, pH 5·8 (the optimum pH under these conditions). Tris–acetic acid buffer strongly enhances enzyme activity at the lower substrate concentrations but only slightly at higher concentrations.

The optimum substrate concentration for glycosulphatase acting on glucose 3-*O*-sulphate is in the region of 0·07 M, whereas, at the same concentration of galactose 6-*O*-sulphate, maximum enzyme activity has still not been achieved. Table 2 lists the Michaelis constants (calculated from the plot of $[S]/v$ against $[S]$; Lineweaver & Burk, 1934), the optimum pH and the relative activity of the enzyme towards the three substrates. The enzyme shows greatest affinity and activity towards glucose 6-*O*-sulphate. Fig. 2 shows the effect of pH on enzyme activity towards the three substrates in the presence of tris–acetic acid buffer.

Effect of time of incubation and enzyme concentration. The results of these experiments are shown in

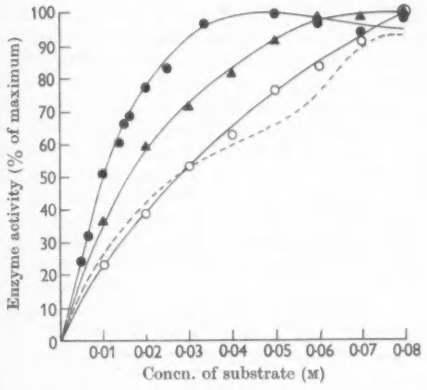

Fig. 1. Substrate concentration–activity curves for *Littorina* glycosulphatase acting on potassium glucose 6-*O*-sulphate, potassium glucose 3-*O*-sulphate and potassium galactose 6-*O*-sulphate in the presence of 0·5 M-tris–acetic acid buffer at the appropriate optimum pH. ●, Potassium glucose 6-*O*-sulphate, pH 5·5; ▲, potassium glucose 3-*O*-sulphate, pH 5·8; ○, potassium galactose 6-*O*-sulphate, pH 5·2. The broken line shows the corresponding curve for the same concentration of enzyme acting on potassium glucose 6-*O*-sulphate in the presence of 0·5 M-sodium acetate–acetic acid, pH 5·8.

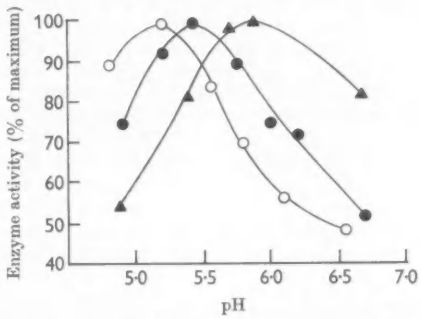

Fig. 2. Effect of pH on the activity of *Littorina* glycosulphatase towards potassium glucose 6-*O*-sulphate, potassium glucose 3-*O*-sulphate and potassium galactose 6-*O*-sulphate in the presence of 0·5 M-tris–acetic acid buffer. ●, 0·04 M-Potassium glucose 6-*O*-sulphate; ▲, 0·04 M-potassium glucose 3-*O*-sulphate; ○, 0·04 M-potassium galactose 6-*O*-sulphate.

Table 2. *Michaelis constants* (K_m), pH *optima, substrate-concentration optima and relative activities under optimum conditions for the glycosulphatase of* Littorina littorea *acting on the* 3-O- *and* 6-O-*sulphates of glucose and the* 6-O-*sulphate of galactose in the presence of* 0·5 M-*tris–acetic acid buffer*

Substrate	Optimum pH	$10^2 \times$ Optimum substrate concn. (M)	$10^2 K_m$	Relative activity
Potassium glucose 3-*O*-sulphate	5·7–5·9	7·0	3·0	1·0
Potassium glucose 6-*O*-sulphate	5·5–5·6	4·0	1·7	10·0
Potassium galactose 6-*O*-sulphate	5·2	> 8·0	7·2	2·1*

* Measured at a substrate concentration of 80 mM.

Fig. 3. Glucose 6-*O*-sulphate was used as substrate at a final concentration of 0·04 M in 0·5 M-tris–acetic acid buffer, pH 5·5. Enzyme activity did not decline with time over a period of 70 min. and the enzyme concentration–activity curve provided no evidence for the presence of endogenous activators or inhibitors.

Effect of buffer concentration on glycosulphatase activity. Variation of the concentration of sodium acetate–acetic acid buffer, pH 5·8, between the limits 0·25 and 1·0 M had little effect on enzyme activity towards 0·04 M-glucose 6-*O*-sulphate. On the other hand, with tris–acetic acid buffer, pH 5·5, and the same concentration of substrate, there was a linear increase in enzyme activity with buffer concentration (overall increase of 15% between 0·25 and 1·0 M).

Effect of various compounds on enzyme activity. Table 3 shows the effects of some common enzyme inhibitors and activators on enzyme activity towards 0·04 M-glucose 6-*O*-sulphate in the presence of 0·5 M-tris–acetic acid buffer, pH 5·5. In common with most other sulphatase enzymes, glycosulphatase was strongly inhibited by phosphate and pyrophosphate and to a less extent by fluoride. Some increase in enzyme activity was observed in the presence of $MgCl_2$ and $MnCl_2$.

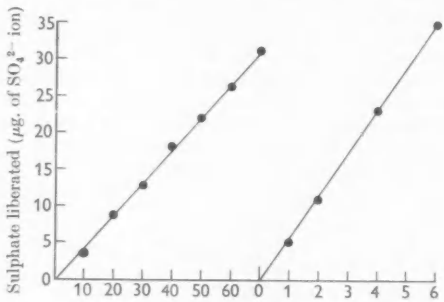

Fig. 3. Effects of time of incubation and relative enzyme concentration on the activity of *Littorina* glycosulphatase towards 0·04 M-potassium glucose 6-*O*-sulphate in the presence of 0·5 M-tris–acetic acid, pH 5·5.

Table 3. *Effects of various compounds on the activity of the glycosulphatase of* Littorina littorea

Results are expressed as a percentage of the enzyme activity obtained towards potassium glucose 6-*O*-sulphate (0·04 M in 0·5 M-tris–acetic acid buffer, pH 5·5) in the absence of added activator or inhibitor. Except where otherwise stated, the final concentration of inhibitor or activator was 0·02 M. All inhibitor and activator solutions were adjusted to pH 5·5 with a trace of M-acetic acid or -NaOH before use.

Compound added	Enzyme activity (%)
KCN	110
NaF	27
NaCl	102
$MgCl_2$	112
$MnCl_2$	120
$ZnCl_2$	88
NaH_2PO_4–Na_2HPO_4	3
$Na_4P_2O_7$*	1
Hydroxylamine	91
Semicarbazide	71
Sodium ethylenediaminetetra-acetate	104

* Final concentration 0·01 M.

*Activity of the enzyme preparation towards sodium cortisone 21-sulphate and sodium dehydro*iso*-androsterone sulphate*

The final enzyme preparation still retained activity towards cortisone 21-sulphate and dehydro-*iso*androsterone sulphate, and Figs. 4 and 5 show the effect of substrate concentration and pH on enzyme activity towards these substrates in the presence of 0·5 M-tris–acetic acid and sodium acetate–acetic acid buffers respectively. With dehydro*iso*androsterone sulphate the method of assay did not allow the use of substrate concentrations below 1·5 mM (see Dodgson, 1961). Roy (1956) has noted that the optimum concentration of this substrate for the 3β-steroid sulphatase of *Patella vulgata* is about 0·2 mM. The curve shown in Fig. 4 therefore presumably reflects the inhibition of enzyme activity by excess of substrate.

The probability that different enzymes are responsible for the hydrolysis of dehydro*iso*androsterone sulphate and cortisone 21-sulphate by *Patella* preparations has already been indicated by Roy (1956). This author suggested that glycosulphatase might be the enzyme responsible for the

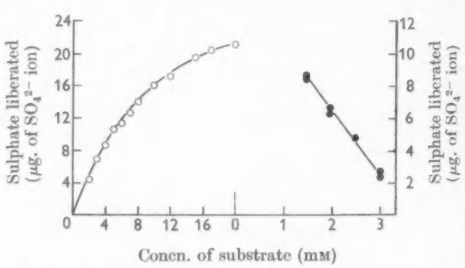

Fig. 4. Substrate concentration–activity curves for *Littorina* preparations (stage 5) acting on sodium cortisone 21-sulphate and sodium dehydro*iso*androsterone sulphate. ○, Cortisone 21-sulphate in the presence of 0·5 M-tris–acetic acid buffer, pH 5·1; ●, dehydro*iso*androsterone sulphate in the presence of 0·5 M-sodium acetate–acetic acid buffer, pH 4·5.

hydrolysis of the cortisone ester. Table 4 shows the relative rates of hydrolysis of glucose 6-*O*-sulphate, cortisone 21-sulphate and dehydro*iso*androsterone sulphate by four separate *Littorina* preparations (stage 5) under standard experimental conditions. The variation in relative activities from preparation to preparation suggests that the three substrates are hydrolysed by different enzymes.

Mixed-substrate experiments. Attempts were made to confirm the existence of three distinct enzymes by mixed-substrate experiments. The theoretical background to this type of experiment has been outlined by Dixon & Webb (1958) and by Laidler (1958). If one enzyme is responsible for the hydrolysis of two different substrates then each will act as a competitive inhibitor of the other when both are present in the same incubation mixture. Overall enzyme activity will therefore be less than the sum of the activities when each substrate is incubated separately with enzyme. In contrast, if two enzymes are involved, the overall activity when two substrates are present should approximate to the sum of the activities obtained when the two substrates are incubated separately with the same amount of enzyme.

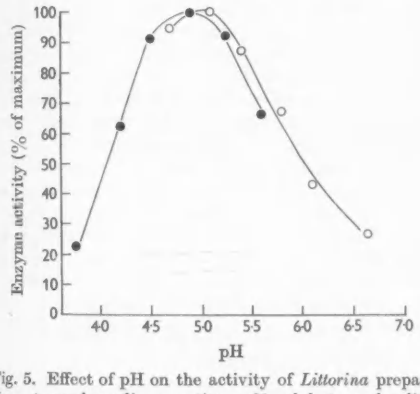

Fig. 5. Effect of pH on the activity of *Littorina* preparations towards sodium cortisone 21-sulphate and sodium dehydro*iso*androsterone sulphate. ○, 0·01 M-Cortisone 21-sulphate in 0·5 M-tris–acetic acid buffer; ●, 2 mM-dehydro-*iso*androsterone sulphate in 0·5 M-sodium acetate–acetic acid buffer.

The following procedure was used in experiments with cortisone 21-sulphate and glucose 6-*O*-sulphate. Each substrate (at a concentration four times as great as that required in the final incubation mixture) was dissolved in 1 M-tris–acetic acid buffer, pH 5·6. A portion of each stock solution was removed and diluted with an equal volume of 1 M-tris–acetic acid buffer, pH 5·6, and a 'mixed-substrate' solution was prepared by taking a further portion of one of the stock solutions and diluting it with an equal volume of the other stock solution. A portion (0·025 or 0·05 ml.) of each of the three substrate solutions thus obtained was incubated with an equal volume of the enzyme preparation (usually diluted two- or three-fold with water) for 1 hr. at 38°. Liberated SO_4^{2-} ion was then estimated in the usual way, except that the trichloroacetic acid contained 12 μg. of SO_4^{2-} ion (as K_2SO_4)/ml. A similar procedure was used with other substrate combinations except that the conditions of buffer and pH were sometimes different. In all cases, preliminary experiments were made in order to check that SO_4^{2-} ion could be estimated under the experimental conditions used.

Table 5 records the results of experiments in which the combinations of substrates were glucose 6-*O*-sulphate and cortisone 21-sulphate, dehydro-*iso*androsterone sulphate and cortisone 21-sulphate, and dehydro*iso*androsterone sulphate and glucose 6-*O*-sulphate. The amount of SO_4^{2-} ion liberated from glucose 6-*O*-sulphate and cortisone 21-sulphate, when both are present in the same incubation mixture, agrees very closely with the sum of the amounts liberated when each substrate is incubated separately, and this is good evidence that the two substrates are hydrolysed by different enzymes. Similar conclusions can be drawn for glucose 6-*O*-sulphate and dehydro*iso*androsterone sulphate. On the other hand, the results with dehydro*iso*androsterone sulphate and cortisone 21-sulphate suggest that the same enzyme is attacking both substrates. However, Dixon & Webb (1958)

Table 4. *Variation in the relative activities of four different* Littorina *preparations towards glucose 6-O-sulphate, dehydro*iso*androsterone sulphate and cortisone 21-sulphate*

Experimental conditions were: cortisone 21-sulphate (CS), 0·01 M in 0·5 M-tris–acetic acid, pH 5·1; dehydro-*iso*androsterone sulphate (DHAS), 2 mM in 0·25 M-sodium acetate–acetic acid, pH 4·5; glucose 6-*O*-sulphate (GS), 0·04 M in 0·5 M-tris–acetic acid, pH 5·6.

Enzyme preparation	Date of collection of organisms	Relative activity towards CS	DHAS	GS
1	13 Oct. 1959	4·3	1·0	9·9
2	26 Oct. 1959	2·4	1·0	3·8
3	5 Nov. 1959	3·5	1·0	3·8
4	4 Apr. 1960	3·3	1·0	6·3

state: 'When additive effects are obtained, it is good evidence that two independent enzymes are concerned, but less-than-additive effects are not conclusive evidence for identity, since each enzyme might be competitively inhibited by the substrate of the other'. Inhibition of this type could well explain the experimental results obtained with dehydro*iso*androsterone sulphate and cortisone 21-sulphate.

Observations with other enzyme preparations. The digestive juice of the snail, *Helix pomatia*, contains 3β-steroid sulphatase (Henry & Thevenet, 1952), and attempts were made to test the ability of this juice to hydrolyse glucose 6-*O*-sulphate and cortisone 21-sulphate. Unpublished observations from these laboratories have shown that incubation of the juice alone results in the liberation of small amounts of SO_4^{2-} ion from endogenous sulphated

Table 5. *Results of 'mixed-substrate' experiments with* Littorina *and* Helix *preparations*

See text for explanation of these experiments. The amount of SO_4^{2-} ion liberated enzymically when two substrates are present in the same incubation mixture should be compared with the sum (figures in parentheses) of the amounts liberated when the same two substrates are incubated separately. The following abbreviations are used: CS, cortisone 21-sulphate; GS, glucose 6-*O*-sulphate; DHAS, dehydro*iso*androsterone sulphate.

Enzyme	Substrate present in the incubation mixture	Concn. of substrate in incubation mixture (mM)	Buffer	pH	SO_4^{2-} ion liberated (μg.) From 'mixed substrate'	SO_4^{2-} ion liberated (μg.) From individual substrates
Littorina littorea	CS	20	0·5M-Tris–acetic acid	5·6	—	11·9
	GS	40			—	12·6
	CS and GS	—			23·6	(24·5)
	CS	20	0·5M-Tris–acetic acid	5·6	—	12·2
	GS	60			—	13·6
	CS and GS	—			23·8	(25·8)
	CS	10	0·5M-Tris–acetic acid	5·1	—	13·2
	DHAS	2			—	8·5
	CS and DHAS	—			11·6	(21·7)
	CS	10	0·5M-Sodium acetate–acetic acid	5·1	—	18·3
	DHAS	2			—	13·2
	CS and DHAS	—			11·4	(31·5)
	DHAS	2	0·5M-Tris–acetic acid	5·1	—	7·2
	GS	20			—	13·3
	DHAS and GS	—			19·7	(20·5)
Helix pomatia (digestive juice)	CS	10	0·5M-Sodium acetate–acetic acid	5·1	—	17·8
	DHAS	2			—	1·4
	CS and DHAS	—			11·9	(19·2)

Table 6. *Activity of some* Helix *preparations towards cortisone* 21-*sulphate, dehydro*isoandrosterone *sulphate, glucose* 6-O-*sulphate and galactose* 6-O-*sulphate*

Experimental conditions were: cortisone 21-sulphate (CS), 0·01M, in 0·5M-tris–acetic acid buffer, pH 5·1; dehydro*iso*androsterone sulphate (DHAS), 2 mM in 0·5M-sodium acetate–acetic acid buffer, pH 4·5; glucose 6-*O*-sulphate (GS), 0·04M in 0·5M-tris–acetic acid buffer, pH 5·5; galactose 6-*O*-sulphate (Gal.S), 0·04M in 0·5M-tris–acetic acid buffer, pH 5·2. The digestive-juice preparations were diluted 40-fold before use and the digestive-gland arylsulphatase preparation (Dodgson & Powell, 1959) was used without further dilution. Incubation mixtures (0·1 ml.) contained 0·05 ml. of enzyme solution and incubation was for 2 hr. at 38°.

Enzyme preparation	SO_4^{2-} ion (μg.) liberated by 0·05 ml. of enzyme in 2 hr. from: CS	DHAS	GS	Gal.S
Digestive juice (July)	28·6	<1·0 <1·0*	0	0
Digestive juice (Sept.)	36·7	1·1 1·5*	0	0
Digestive-gland arylsulphatase preparation	13·4	<1·0	0	—

* Incubation mixture consisted of 0·1 ml. of enzyme and 0·1 ml. of a 1·2 mM-solution of dehydro*iso*androsterone sulphate in 0·8M-sodium acetate–acetic acid buffer, pH 4·5, and liberated SO_4^{2-} ion was measured by the chloranilate procedure of Lloyd (1959). Results are corrected to 0·05 ml. of enzyme solution.

materials. Before use, therefore, the juice was incubated for 24 hr. at 38° in the presence of sodium acetate–acetic acid buffer (final concn. approx. 0·15 M), pH 5·6, and subsequently dialysed at 4° for 24 hr. against several changes of water.

The treated juice was usually diluted (with water) 30- or 40-fold for the enzyme experiments. Enzyme activity was measured in the usual way (Dodgson, 1961), except that with one of the two preparations used, incubation with dehydro*iso*androsterone sulphate resulted in the appearance of a faint turbidity. This turbidity did not appear in the controls where enzyme and substrate were incubated separately. Further controls were necessary to compensate for this turbidity and in practice these consisted of an additional series of test and control determinations in which a 0·5 % solution of gelatin was substituted for the $BaCl_2$–gelatin reagent during the estimation of the liberated SO_4^{2-} ion. The difference in the spectrophotometric readings (usually approx. 0·025) between these additional tests and controls was deducted from that obtained when the normal test solutions were measured against the normal controls.

Table 6 records the relative activities of two separate enzyme preparations (obtained from snails collected in July and September respectively) towards glucose 6-*O*-sulphate (0·04 M, in 0·5 M-tris–acetic acid buffer, pH 5·5), galactose 6-*O*-sulphate (0·04 M, in 0·5 M-tris–acetic acid, pH 5·2), dehydro*iso*androsterone sulphate (2 mM, in 0·5 M-sodium acetate–acetic acid buffer, pH 4·5) and cortisone 21-sulphate (0·01 M, in 0·5 M-tris–acetic acid buffer, pH 5·1). Cortisone 21-sulphate was rapidly hydrolysed by both preparations but activity towards dehydro*iso*androsterone sulphate was very low. 'Mixed-substrate' (dehydro*iso*androsterone sulphate and cortisone 21-sulphate) experiments gave results (Table 5) which were similar to those obtained with the *Littorina* preparation. No enzyme activity towards the 6-*O*-sulphates of glucose and galactose could be detected in either *Helix* preparation. It was possible that endogenous factors were inhibiting glycosulphatase activity but separate experiments showed that no significant reduction in the activity of *Littorina* glycosulphatase occurred when the *Helix* preparation was also present in the incubation mixture.

Further experiments were made with a partially purified preparation of the arylsulphatase of the digestive gland of *Helix* (see Dodgson & Powell, 1959). This preparation again exhibited appreciable activity towards cortisone 21-sulphate but little or no activity towards dehydro*iso*androsterone sulphate or glucose 6-*O*-sulphate (Table 6).

Table 7. *Sulphate esters which were not hydrolysed by* Littorina *or* Helix *preparations*

In most cases incubation mixtures consisted of 0·05 ml. of enzyme solution (diluted threefold for *Littorina* and 30-fold for *Helix*) and an equal volume of a 0·08 M-solution of the sulphate ester in 1 M-tris–acetic acid buffer, pH 5·5. Sulphate esters of hydroxylated amino acids were used at a concentration of 0·04 M in the same buffer at pH 5·7 and 8·5. Incubation periods varied between 16 and 24 hr. The sulphate esters were added as the potassium salts.

Methyl sulphate	*cyclo*Hexyl sulphate
Ethyl sulphate	Tetrahydrofuran 2-methyl sulphate
Ethanolamine sulphate	Tetrahydropyran 2-methyl sulphate
*iso*Propyl sulphate	Propane-1:3-diol disulphate
1-Aminopropan-2-ol sulphate	Serine *O*-sulphate
n-Butyl sulphate	Threonine *O*-sulphate
sec.-Butyl sulphate	Hydroxyproline *O*-sulphate
n-Pentyl sulphate	
n-Hexyl sulphate	
*cyclo*Pentyl sulphate	

Activity of Littorina *and* Helix *preparations towards other sulphate esters*

Littorina and *Helix* (digestive juice) preparations were without effect on the sulphate esters listed in Table 7. The various experimental conditions used are also recorded in this table. The results confirm the findings of other workers (e.g. Roy, 1956) that mollusc preparations do not contain a general 'alkylsulphatase'.

DISCUSSION

Previous work from these and other laboratories has shown the potential value of enzymes as tools in the elucidation of the structures of sulphated polysaccharides (see Dodgson & Lloyd, 1958; Takahashi, 1960*a*). Glycosulphatase may be of some use in this type of study, and one of the aims of the present work has been to obtain preparations of glycosulphatase which are relatively free from other enzymes which might be active towards such polymers. The purification procedure described here results in the elimination of the chondroitinase (aminopolysaccharase) and most of the β-glucuronidase and β-*N*-acetylglucosaminidase which are initially present in *Littorina* extracts and which can participate in the degradation of chondroitin sulphates A and C. The resulting preparation is quite stable when stored in the frozen state and can be regarded as suitable starting material for further purification.

Littorina glycosulphatase is particularly active towards glucose 6-*O*-sulphate but shows relatively low activity towards glucose 3-*O*-sulphate and galactose 6-*O*-sulphate. Earlier work (Soda, 1936), in which relatively crude and ill-defined preparations of various carbohydrate sulphate esters were used, indicated that the glycosulphatase of *Charonia lampas* also showed greatest activity towards a substrate preparation now known to

have consisted mainly of glucose 6-*O*-sulphate. Recently, Takahashi (1960*b*) has purified the *Charonia* enzyme 160-fold by chromatography on carboxymethylcellulose columns. The purified enzyme exhibits properties which are very similar to those of the *Littorina* enzyme.

The anomalies in the substrate concentration–activity curve obtained when the enzyme is acting on glucose 6-*O*-sulphate in the presence of sodium acetate–acetic acid buffer (Dodgson & Lloyd, 1961) do not appear with tris–acetic acid buffers. Other partially purified sulphatase enzymes exhibit similar anomalies in the presence of sodium acetate–acetic acid buffers (see Dodgson & Wynn, 1958). It is probable that these effects merely reflect the comparative impurity of the enzyme preparations since in one case (human-liver arylsulphatase B, Dodgson & Wynn, 1958) the anomalies disappeared on further purification.

The finding of sulphatase activity towards cortisone 21-sulphate in *Patella* (Roy, 1956), *Otala* (Savard, Bagnoli & Dorfman, 1954), *Littorina* and *Helix*, the only organisms examined up to the present time, may well point to a widespread distribution of such activity in molluscs. The present work shows that the enzyme responsible for the hydrolysis of this substrate cannot be identified with glycosulphatase or chondrosulphatase. The results of 'mixed-substrate' experiments with dehydro*iso*androsterone sulphate and cortisone 21-sulphate, although suggesting that one enzyme acts on both substrates, can also be interpreted as arising from competitive inhibition of each of two distinct enzymes by the substrate of the other. The variation in the relative activities towards dehydro*iso*androsterone sulphate and cortisone 21-sulphate exhibited by *Patella*, *Littorina* and *Helix* preparations (see Roy, 1956, and Tables 4 and 6) strongly suggests that two different enzymes are involved. Roy (1956) has already pointed out that the high specificity of 3β-steroid sulphatase for the 3β-sulphate esters of 5α- and Δ^5-steroids makes it unlikely that the enzyme could be responsible for the hydrolysis of cortisone 21-sulphate.

Arylsulphatase (Dodgson, Lewis & Spencer, 1953; Dodgson & Powell, 1959), cellulose polysulphatase (Takahashi & Egami, 1960) and myrosulphaminase (myrosulphatase; see Takahashi, 1960*c*) also occur in some molluscs, and the possibility that these enzymes can attack cortisone 21-sulphate cannot yet be discounted. However, with *Patella* concentrates Roy (1956) has obtained some degree of separation of arylsulphatase from the enzyme hydrolysing cortisone 21-sulphate, by means of paper electrophoresis. Cellulose polysulphatase is known to be only slightly inhibited by 0·02 M-sodium fluoride (Takahashi & Egami, 1960), whereas enzyme activity towards cortisone 21-sulphate is completely inhibited under these conditions (K. S. Dodgson, unpublished observations). Myrosulphaminase, an enzyme liberating sulphuric acid from mustard-oil glycosides, is concerned with the rupture of a N–O–S linkage and from theoretical grounds would hardly be expected to inhibit activity towards substrates containing C–O–S linkages.

Leon, Bulbrook & Corner (1960) have reported that some molluscs (including *Helix*) possess sulphatase activity towards aetiocholanolone sulphate which cannot be attributed to 3β-steroid sulphatase. However, this enzyme was not present in *Littorina*. The probability exists therefore that the enzyme acting on cortisone 21-sulphate is a new type of sulphatase, but much work remains to be done before this can be established with certainty.

The digestive juice of *Helix pomatia* is extremely active towards cortisone 21-sulphate. At pH 5·1, in the presence of 0·5 M-tris–acetic acid, the activity towards 0·01 M-cortisone 21-sulphate of the more active of the two preparations tested corresponded to a liberation of approx. 43 000 μg. of SO_4^{2-} ion/ml. of undiluted preparation/hr.

SUMMARY

1. The glycosulphatase of the digestive organs of the common periwinkle, *Littorina littorea*, has been purified 20-fold. Chondroitinase, β-glucuronidase and β-*N*-acetylglucosaminidase, which are also present in aqueous extracts of the digestive organs, are eliminated or greatly decreased in concentration during the purification procedure.

2. The potassium salts of the 3-*O*-sulphate and 6-*O*-sulphate esters of glucose and the 6-*O*-sulphate esters of galactose have been used as substrates for glycosulphatase. The enzyme shows greatest affinity and activity towards glucose 6-*O*-sulphate.

3. The final enzyme preparation, which also contains appreciable amounts of arylsulphatase and 3β-steroid sulphatase, is able to hydrolyse sodium cortisone 21-sulphate. Mixed-substrate experiments provide no support for the suggestion (Roy, 1956) that glycosulphatase is the enzyme responsible for the hydrolysis of cortisone 21-sulphate.

4. Preparations of the digestive juice of *Helix pomatia*, when diluted 40-fold, show little or no activity towards glucose 6-*O*-sulphate and dehydro*iso*androsterone sulphate but are strongly active towards cortisone 21-sulphate.

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The Occurrence of Unusual Fatty Acids in Faecal Lipids from Human Beings with Normal and Abnormal Fat Absorption

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Little information has been published on the detailed fatty acid composition of human faecal lipids, though it has long been a vexed question whether or not the microbial flora of the intestinal tract play a major part in the production of the large amounts of fat excreted in diseases such as steatorrhoea or sprue. Studies relating the composition of faecal lipids to that of the ingested fat should provide some information on this problem.

The earlier methods of fatty acid analysis used in such studies, e.g. fractional distillation (Edwards & Cook, 1951) and reversed-phase liquid–liquid chromatography (Van de Kamer, Pikaan, Bolssens-Frankena, Couvée-Ploeg & van Ginkel, 1955), are not capable of providing sufficiently detailed information. We have used gas–liquid chromatography both for analysis (James & Martin, 1956) and for structure determination (James & Webb, 1957; James, 1959).

EXPERIMENTAL

Faeces were collected and either extracted immediately with the medium described by Dole (1956) or stored at 2° until required. The extracted lipids were divided into free and bound acids by the titration technique described by Dole (1956).

The free acids were methylated directly with anhydrous methanolic HCl (James, Lovelock, Webb & Trotter, 1957) and the bound acids were methylated after saponification and extraction. Analyses of the methyl esters of the fatty acids were carried out on 80–100 μg. samples on 4 ft. glass columns with stationary phases of Apiezon L grease (Shell Chemicals Ltd.) at 200° with a column efficiency of 4000–5000 theoretical plates, and also polyethylene glycol adipate at 180° with an efficiency of 3000–3500 theoretical plates. In both cases the argon ionization monitor was used as detector (Lovelock, 1958; Lovelock, James & Piper, 1959; and Pye Scientific Instruments Ltd.). Isolation of individual acids on the milligram scale was carried out on similar columns with the gas-density meter as

detector (James & Webb, 1957). Identification of individual acids was carried out by measurement of log relative retention volumes on the two types of column described by James (1959). Determinations of structure of unsaturated and hydroxy acids were done by the permanganate-oxidation method with gas–liquid chromatography to identify the mono- and di-carboxylic acids produced (James & Webb, 1957).

The u.v. absorptions of isolated acids were determined at a concentration of 1 mg./ml. in purified *n*-hexane at a wavelength range of 250–315 mμ. Infrared spectra were determined by the KCl-disk technique. *Trans*-acid contents were determined in CS_2 solution by the method described by Hartman, Shorland & Cleverley (1958). A standard curve was constructed with known concentrations of methyl elaidate; solutions of methyl stearate at similar concentrations were used as the solvent blank.

Faecal lipids were separated into different classes with the silicic acid chromatogram described by Hirsch & Ahrens (1958).

RESULTS

Fig. 1 shows a typical chromatogram of the faecal fatty acids of a human subject with steatorrhoea (a heavier-than-normal load being used to demonstrate the minor components). A qualitatively similar picture is given by a normal subject. Two major peaks can be seen in unusual positions: (*a*) in a position expected for positional and configurational isomers of oleic acid and (*b*) in a position expected for a C_{20} monoenoic acid.

Identification of the substances responsible for these peaks was carried out as described below.

Peak (a). On running the same mixture as in Fig. 1 on a polyethylene glycol adipate column, peak (*a*) moved into the oleic acid peak, behaviour expected from isomeric octadecenoic acids on this type of column. A sample of the components in

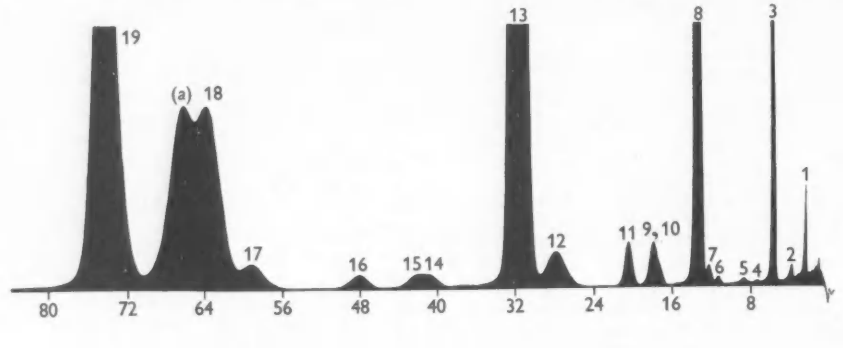

Fig. 1. Gas chromatogram of methyl esters of the fatty acids isolated from human faecal lipids, a heavy load being used to demonstrate the minor components. Conditions: column load, 250 μg.; temperature, 197°; argon inlet pressure, 15 lb./in.^{2} above atmosphere, outlet at atmospheric pressure; stationary phase, 15% (w/w) Apiezon L vacuum grease; support, Celite (100–120 mesh); column length, 4 ft. Apparatus by W. G. Pye Ltd., Cambridge. Detector, argon ionization monitor. Peaks in order of appearance: (1) methyl *n*-decanoate; (2) methyl *n*-undecanoate; (3) methyl *n*-decanoate; (4) branched tridecanoate; (5) methyl *n*-tridecanoate; (6) highly branched tetradecanoate; (7) methyl myristoleate; (8) methyl *n*-tetradecanoate; (9), (10) branched pentadecanoates; (11) methyl *n*-pentadecanoate; (12) methyl palmitoleate; (13) methyl *n*-hexadecanoate; (14), (15) branched heptadecanoates; (16) methyl *n*-heptadecanoate; (17) methyl linoleate; (18) methyl oleate; (*a*) unknown; (19) methyl *n*-octadecanoate, (*b*) unknown.

Table 1. *Fragments isolated from oxidative degradation of the oleic acid-isomer peak*

Monocarboxylic acid	Dicarboxylic acid	Parent unsaturated acid	Approximate amount as percentage of total peak
C_6	C_{12}	Δ^{12}-Octadecenoate	3·5
C_7	C_{11}	Δ^{11}-Octadecenoate	45·5
C_8	C_{10}	Δ^{10}-Octadecenoate	18·1
C_9	C_9	Δ^9-Octadecenoate	17·0
C_{10}	C_8	Δ^8-Octadecenoate	6·6
Masked by C_{11} carrier	C_7	Δ^7-Octadecenoate	3·6
C_{12}	C_6	Δ^6-Octadecenoate	2·8
C_{13}	C_5	Δ^5-Octadecenoate	1·4
Trace of C_{14}	C_4	Δ^4-Octadecenoate	0·9

Table 2. Trans-*acid content of faecal lipids*

Sample	Percentage of *trans*-acid determined spectroscopically	Percentage of oleic acid isomers in sample
Mixed faecal acids	10·1	7·6
Equimolar mixture of isolated oleic acid and oleic acid isomers	26	50

peak (*a*) was isolated with an Apiezon L column, and oxidative degradation followed by identification of the mono- and di-carboxylic acids produced gave the results shown in Table 1. The components of peak (*a*) therefore consist of a mixture of Δ^4-, Δ^5-, Δ^6-, Δ^7-, Δ^8-, Δ^9-, Δ^{10}-, Δ^{11}- and Δ^{12}-octadecenoic acids. The relative amounts of the different acids are also given in Table 1, the major components being the Δ^8-, Δ^9-, Δ^{10}- and Δ^{11}-octadecenoic acids, the last-named representing 45% of the mixture.

Determination of the *trans*-acid content (see Experimental section) of the total mixed faecal acids gave a value of 10% (Table 2), the same order as the amount of oleic acid isomers in the sample (7·6%). However, estimation of the *trans*-acid content of an approximately equimolar mixture of oleic acid and its isomers isolated from the mixed faecal acids gave a value of only 26%, suggesting that only about 50% of the oleic acid isomers possess the *trans*-configuration, and that other acids in the mixed sample are also *trans*-acids.

Peak (b). Although the change in position of peak (*a*) on the polyethylene glycol adipate column was readily observed, no peak could be detected in the position expected for a C_{20} monoenoic acid (the suggested structure for peak *b*). Only after prolonging the time of analysis was a peak found agreeing in area with the expected for peak (*b*); its retention relative to methyl stearate was 9·0. The substance responsible for this peak was isolated and run again on the Apiezon L column and had a retention volume relative to methyl stearate of 1·96.

When log relative retention volumes of a variety of acids in the Apiezon L column are plotted against the values obtained in the polyethylene glycol adipate column, a family of parallel lines can be drawn through the points belonging to different classes of fatty acids, i.e. saturated, mono-, di-, tri-, tetra-, penta- and hexa-enoic acids (James, 1959). The values obtained for peak (*b*) did not fall on any of these lines and suggested an impossible number of double bonds in the molecule. Examination of the u.v.-absorption spectrum of a sample of the unknown acid dissolved in spectroscopic-quality *n*-hexane failed to reveal any absorption at the wavelengths expected for unsaturated acids (250–315 mμ). An attempt was made to reduce catalytically a sample of the acid under pressure, but only the original material could be isolated. Thus the unknown acid possessed some unusual group or groups not found in the common straight-chain saturated or unsaturated acids. The presence of a ring structure in the unknown acid was possible, so lactobacillic acid was tried on the two columns but did not show a large enough relative retention in the polar column (Table 3). This idea was not further pursued. Since the London dispersion forces between solute and solvent are of the same order in both the polyester and Apiezon L columns, the large retention of the unknown acid in the polyester column must be due to some very polar group in the molecule. The effect of an hydroxyl group on chromatographic behaviour was not known so ricinoleic acid (12-hydroxy-Δ^9-octadecenoic acid) was chosen as a model substance. This compound has a relative retention volume of 9·05 in the Apiezon L column whereas oleic acid (Δ^9-octadecenoic acid) had a relative retention of 4·75. The ratio of these two figures gave a relative retention of 1·9 for the hydroxyl group alone (Table 3). A pure sample of methyl ricinoleate was isolated by means of the gas chromatogram and catalytically reduced to give 12-hydroxystearic acid. This

Table 3. *Relative retention volumes (methyl myristate = 1) of methyl esters of saturated, unsaturated and hydroxy acids in two stationary phases*

Acid	Relative retention volumes: Apiezon L at 197°	Relative retention volumes: Polyethylene glycol adipate at 180°
Oleic (*cis*-Δ^9-octadecenoic)	4·75	4·42
Elaidic (*trans*-Δ^9-octadecenoic)	4·95	4·42
cis-Δ^4-Octadecenoic	4·95	—
trans-Δ^4-Octadecenoic	5·15	—
Oleic isomers (from faeces)	4·95	4·42
Lactobacillic	7·22	8·60
Ricinoleic (12-hydroxy-Δ^9-octadecenoic)	9·05	—
12-Hydroxystearic	11·0	36·0
10-Hydroxystearic (from faeces)	11·0	35·0
DL-10-Hydroxystearic (synthetic)	11·0	35·7
9:10-Dihydroxystearic	21·2	Too slow to measure
Stearic	5·5	4·0
Branched pentadecanoic (possibly *iso*-)	1·3	1·19
Branched pentadecanoic (possibly *anteiso*)	1·35	1·27
Branched heptadecanoic (possibly *iso*-)	3·14	2·55

Table 4. *Acids obtained from oxidative degradation of the hydroxystearic acid*

Dicarboxylic acids	Monocarboxylic acids	Corresponding hydroxy acid
C_4	C_4 (trace)	6-Hydroxystearic acid
C_5	C_{13} (trace)	7-Hydroxystearic acid
C_6	C_{12}	8-Hydroxystearic acid
C_7	C_{11}	9-Hydroxystearic acid
C_8	C_{10}	
C_9*	C_9*	10-Hydroxystearic acid
C_{10}*	C_8*	

* Major components.

Table 5. *Comparison of composition of esterified (bound) and non-esterified (free) fatty acids in faecal lipids for a normal subject and a steatorrhoea subject on a general ward diet*

Trace: less than 0·5%.

Fat excretion	Percentage of acids in range C_6–C_{20}: Normal (G)		Steatorrhoea (W.S.)	
In 24 hr. (g.)	< 5		17·7	
Acid	Free	Bound	Free	Bound
10:0	0·6	0	0·8	Trace
12:0	4·3	2·3	8·2	2·4
14:0	8·9	4·4	11·9	6·2
Branched 15:0	0·7	1·1	0·4	0·3
15:0	1·4	1·1	0·5	0·4
16:0	55·2	35·3	31·7	41·1
Branched 17:0	0·9	1·4	0·5	0·6
17:0	0·4	0·8	0·3	14·5
18:0	12·9	31·8	5·9	14·5
10-Hydroxy-18:0	0·7	0·9	17·8	2·7
14:1	0	0	0·5	0·2
16:1	1·4	2·1	1·8	1·5
18:1	6·8	10·7	4·1	12·6
Isomer 18:1	3·5	6·5	7·8	11·7
18:2	1·3	2·8	5·9	3·5
18:3	0	0	0	0
20:3	Trace	Trace	4·7	0·9
20:4	Trace	Trace	0·2	Trace
Other unsat. C_{20} acids	Trace	Trace	1·2	1·2

substance behaved on both columns in a manner almost identical with the unknown acid, confirming that this was an hydroxystearic acid.

Comparison of the infrared spectra of the isolated hydroxy acid and 12-hydroxystearic acid showed them to be almost identical, confirming the assigned structure.

Oxidative degradation (James & Webb, 1957) of an isolated sample of the unknown acid gave as major components almost equimolar amounts of the C_8 and C_9 monocarboxylic acids and the C_9 and C_{10} dicarboxylic acids as well as smaller amounts of other monocarboxylic and dicarboxylic acids (Table 4). It was already known that saturated hydroxy acids on oxidation can split on either side of the carbon atom bearing the hydroxyl group (Shukow & Schestakow, 1903). Thus the major component in the unknown acid was 10-hydroxystearic acid, with 6-, 7-, 8- and 9-hydroxystearic acids as minor components.

The small peaks lying between the major components (Fig. 1; peaks nos. 3, 8, 13, 18) were shown to be saturated acids by running on the two types of column. From their chromatographic positions they were identified as *n*-tridecanoic acid, *n*-pentadecanoic acid, *n*-heptadecanoic acid and incompletely resolved branched-chain odd-numbered acids (Table 3). These branched-chain acids are probably the *iso*- and *anteiso*-acids known to occur in animal fats (Hawke, Hansen & Shorland, 1959). Their occurrence in faecal lipids may be due to ingestion of butter, since they are quantitatively increased on a mixed or butter diet as compared with a corn-oil diet.

A total analysis of faecal fatty acids in a normal subject and in a subject with steatorrhoea is given in Table 5. Separation of the faecal lipids into free and bound fatty acids was not very successful by the Dole (1956) technique since a large part of the faecal lipid consists of calcium soaps of the

Table 6. *Composition of the fatty acid content of faecal (steatorrhoea) lipid fractions extracted before and after acidification*

	Percentage of acids in range C_6–C_{20}					
	Light-petroleum extract					
		After separation				Ether extract after acidification
Acid	Before separation	Cholesterol	Triglycerides	Diglycerides	Free acids	
n-Decanoic	0·2	0·2	0·2	0·4	0	0·05
n-Dodecanoic	1·9	1·6	1·0	2·7	0·2	0·29
n-Tetradecanoic	9·1	7·4	4·2	11·0	0·6	4·33
n-Hexadecanoic	25·7	44·3	17·8	11·8	2·0	38·9
n-Octadecanoic	10·5	12·6	5·5	2·7	1·0	39·6
Tetradecenoic	0·2	0·2	0·3	0·2	0	0·04
Hexadecenoic	1·8	2·1	1·4	3·8	0·2	0·26
Oleic	20·4	16·2	18·1	21·2	1·5	3·04
Oleic isomers	6·6	6·6	5·8	2·7	1·0	3·68
Linoleic	3·7	3·6	3·6	7·9	1·1	0·76
C_{20} triene	1·4	Trace	Trace	2·6	3·8	0·20
10-Hydroxystearic	14·0	1·4	35·2	25·3	88	7·04
Fraction as percentage of total extract	—	58	17·7	9·9	11·0	—

Table 7. *Some naturally occurring hydroxy acids*

Structure	Trivial name	Source
12-Hydroxy-Δ^9-octadecenoic	Ricinoleic acid	Castor oil
16-Hydroxyoctadecanoic	Juniperic acid	Castor oil
11-Hydroxyhexadecanoic	Jalapinolic acid	Jalap resin
11-Hydroxypentadecanoic	Convolvulinic acid	Resin from *Convolvulus scammia* L
12-Hydroxydodecanoic	Sabinic acid	Wax from *Juniperus sabina*

Table 8. *Hexadecenoic acids and octadecenoic acids of milk fat and butterfat*

Acid	Configuration	Source	Authors
Δ^9-Hexadecenoic	20% *trans*-	Butterfat	Backderf & Brown (1958)
Δ^{16}-Octadecenoic	*trans*-	Butterfat	
Δ^{11}-Octadecenoic	Mostly *trans*-	Butterfat	
Δ^4-Octadecenoic	Not determined	Milk fat	James & Webb (1957)
Δ^{11}-Octadecenoic	Not determined	Milk fat	
Δ^{13}-Octadecenoic	Not determined	Milk fat	

Table 9. *Effect of the nature of dietary fat on the output of some faecal acids*

		Percentages											
		Palmitic acid		Stearic acid		Oleic acid		Oleic acid isomers		10-Hydroxy-stearic acid		Linoleic acid		Daily output (g.)
	Diet	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	
Normal subject	Low-fat	34·2	28·5	25·4	19·3	17·2	19·5	8·8	10·0	2·0	3·7	2·2	2·1	0·9
	Butter	37·7	44·2	26·4	13·6	6·8	12·7	8·0	10·5	1·1	—	—	2·1	3·0
	Corn oil	17·0	40·8	36·8	16·3	6·7	4·4	25·0	15·8	3·0	10·6	3·3	2·0	4·2
Subject with steatorrhoea	Low-fat	34·2	30·6	6·6	14·4	5·0	8·9	25·7	29·6	—	1·7	1·3	1·99	18·0
	Butter	26·7	30·8	16·3	17·5	18·69	15·8	9·3	8·0	7·3	4·8	3·8	3·0	50·0
	Corn oil	11·7	15·5	6·3	6·2	32·3	31·2	3·9	5·9	3·2	2·1	37·1	36·6	50·0
Composition of dietary fat	Butter	20·8		16·4		24·2		5·0		0		3·0		—
	Corn oil	10·7		3·2		35·0		0		0		50·2		—

fatty acids. To clarify the nature of the faecal lipids, a 24 hr. collection of faeces from a steatorrhoea patient was extracted first with light petroleum to remove most of the bound non-polar lipids and then, after acidification of the faeces with 5N-H_2SO_4, they were extracted with ether. The ether extract readily deposited crystals but the light-petroleum extract, even after removal of the solvent, remained as a greenish oil.

Each extract was resolved into a series of fractions on a silicic acid chromatogram with the sequence of solvents described by Hirsch & Ahrens (1958). The fractions were saponified, and the acids were extracted, esterified and analysed on the gas chromatogram. The results are shown in Table 6. Titration of each of the fractions showed that the second extract was mainly free fatty acid.

Types of lipid in human faeces. Extraction of faeces from a steatorrhoea subject with lipid solvents before and after acidification gave two fractions. The first, largely neutral fat, gave rise to a series of subfractions when subjected to chromatography on silica gel. The material eluted in a zone corresponding to cholesterol esters contained very little hydroxystearic acid (Table 6), whereas eluted in a zone corresponding to triglycerides contained 35·2% of hydroxystearic acid. A later fraction assumed to be free acids contained 88% of the hydroxy acid. This evidence suggested that hydroxystearic acid, though present in ester form, was mainly found in the free faecal acids.

Analysis of the material from the second extraction presumably derived from the calcium soaps present in faeces (Sammons & Wiggs, 1960) gave the result shown in Table 6. This material was shown to be almost entirely non-esterified fatty acid by titration but did not contain more hydroxy acids than did the esterified acids from the first light-petroleum extract. The hydroxy acids are thus found in all the faecal lipids of a steatorrhoea patient, the larger part being in the free acids.

DISCUSSION

The presence of large quantities of hydroxy acids in the faecal lipids of humans with steatorrhoea is of some interest since these acids are not present in any of the normal dietary fats and must therefore be synthesized in the gut. This synthesis is more likely to be carried out by micro-organisms in the gut than in the intestinal wall. Long-chain hydroxy acids are limited in their occurrence in oils and fats but are particularly evident in resins and oils used as cathartics. A list of such acids is given in Table 7. Since the physiological action of castor oil is believed to be due to a hydroxy acid (ricinoleic acid), the diarrhoea often associated with steatorrhoea may be due to production of these acids in the intestine.

The close correspondence of the structure of the hydroxystearic acids with the isomeric octadecenoic acids suggests that they may be derived one from another. At this stage it is impossible to state which is the precursor and which the product, though the hydroxy acids could well be intermediates in the formation of the monounsaturated acids.

The monounsaturated C_{18} acids consist of a mixture of isomers with the double bond varying in position from C_4 to C_{12}, about 50% of the mixture having a *trans*-configuration about the double bond. Some, but not all, of these acids occur in milk and butterfat (see Table 8), so that it is unlikely that they all originate from the diet. In any case, these isomeric acids are still excreted when corn oil is fed; this oil contains only one octadecenoic acid (Table 9).

The occurrence of *trans*-acids in faecal lipids from animals was demonstrated by Hartman, Shorland & Morr (1956) and Hartman *et al.* (1958). These workers [see also Hartman & Shorland (1959)] consider that the *trans*-acids arise by bacterial hydrogenation of polyunsaturated acids in the post-absorptive region of the gut. With the human this hypothesis is unlikely since, in all cases of steatorrhoea studied so far, substitution of corn oil (51% of linoleic acid) for butter (2–3% of linoleic acid) in the diet does not cause any marked increase in the faecal content of the isomeric acids. Indeed, one subject after 3 days on a low-fat diet (less than 12 g./day) was excreting 18 g. of fat/day containing 25·7% of oleic acid isomers in the bound lipids (Table 9). In normal subjects substitution of corn oil for butter in the diet does produce an increase in the content of oleic acid isomers in the faecal fat, but there is also an increase in output of stearic acid that may be serving as the precursor of the unsaturated acids. Subjects with steatorrhoea on a butter diet excrete approximately the same amount of linoleic acid as is ingested, together with large amounts of the *trans*-isomers, again suggesting that these isomers are not produced by hydrogenation of dietary linoleic acid.

SUMMARY

1. Human faecal lipids of subjects both with normal and abnormal fat absorption have a fatty acid composition very different from that of the ingested fats.

2. A variety of isomeric octadecenoic acids are present, with the double bond in positions 4, 5, 6, 7, 8, 9, 10, 11 and 12. Approximately 50% of these oleic acid isomers possess the *trans*-configuration about the double bond.

3. Subjects with steatorrhoea do not excrete increased amounts of *trans*-acids on changing the dietary fat from butter (2–3% of linoleic acid) to corn oil (51% of linoleic acid).

4. On a diet of butter, subjects with steatorrhoea appear to excrete linoleic acid in amounts equal to that ingested.

5. No support is found for the theory that *trans*-octadecenoic acids are produced by bacterial hydrogenation of linoleic acid.

6. A new major component of faecal lipids has been shown to be 10-hydroxystearic acid, together with 6-, 7-, 8- and 9-hydroxystearic acids as minor components.

7. These hydroxy acids are suggested as intermediates in the formation of monounsaturated octadecanoic acids from stearic acid.

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Studies on the Phosphoproteins of Brain

2. PARTIAL PURIFICATION OF A PHOSPHOPROTEIN ATTACHED TO SUBCELLULAR PARTICLES*

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It has been shown that cerebral tissues contain at least two types of phosphoproteins, one attached to particulate matter separable in low centrifugal fields from dispersions of brain made in iso-osmotic sucrose, and the other remaining in the particulate-free supernatant solution (Heald, 1959). The first type is of particular interest, since the incorporation of radioactive phosphate into this phosphoprotein is greatly increased by the brief passage of electrical pulses through slices of cerebral tissue which are actively metabolizing phosphate in the presence of glucose.

To investigate further the systems involved in the phosphorylation and dephosphorylation of this phosphoprotein, it seemed desirable to have available a more purified fraction than was represented by cerebral particles. This paper describes the extraction and partial purification, from such particles, of a lipophosphoprotein. When incubated in a suitable phosphorylating system the purified fraction acts as a phosphate acceptor.

MATERIALS AND METHODS

Tissues. Slices of guinea-pig cerebral cortex were cut and incubated under conditions and in media described by Heald (1959). Ox brains were obtained, as fresh as possible, from the Metropolitan Cattle Market and were packed in a plastic bag surrounded by ice during transportation to the laboratory.

Extraction of phosphoproteins and preparation of a partially purified fraction. Small-scale experiments were performed with samples of guinea-pig brain. Slices were cut and incubated with radioactive phosphate (20–30 μc/slice) for 30–60 min., and were then removed and dispersed with two whole guinea-pig cerebral cortices in 10 vol. of iso-osmotic sucrose (0·25 M), containing 0·5 mM-ethylenediaminetetra-acetate (EDTA), pH 7·4, with a homogenizer similar to that described by Kamphausen & Morton (1956). The dispersion was centrifuged at 1500 *g* for 10 min. and the residue washed twice in the centrifuge with fresh sucrose–EDTA under the same conditions. This residue was then used for testing various extraction procedures. Unincubated cortex was included both to provide sufficient material for separation and in an attempt to dilute any adverse effect of incubation in the medium upon the sedimentation properties of the particles isolated. When a large-scale preparation was made, several such small preparations were stored at −20° and combined to provide sufficient radioactive phosphoprotein to be used as a 'marker' (see below).

Preparations from ox brain. Ox brain from which the medulla, cerebellum and most of the accessible white matter had been removed was freed from meninges and large surface blood vessels, and dispersed, in batches of 36 g., in 10 vol. of iso-osmotic sucrose–EDTA at 2°. Each batch was centrifuged at 1500 *g* for 10 min.; the residue was resuspended in fresh sucrose–EDTA and again centrifuged. The washed residue in iso-osmotic sucrose was stored at 2°. Successive batches were processed until 150 g. wet wt. of tissue had been treated. The combined residues were then mixed with the stored marker fractions (total volume 500 ml.) and to the suspension Teepol L (Shell Chemical Co., Ltd., a mixture of secondary-alkyl sulphates) was added [0·2 ml. (30 mg. of solids)/g. wet wt. of tissue]. After 15–20 min. at room temperature, the lysed suspension was centrifuged at 21 000 rev./min. for 1 hr. in the no. 21 head of the Spinco Model L. The pale-brown supernatant (fraction II) was carefully decanted or pipetted from the white viscid mass at the bottom of the tube. At this point the pH was 7·2. To the supernatant (volume 400 ml.) was added sodium phosphate buffer (M, pH 7·2), 2·0 ml. for each 100 g. wet wt. of tissue taken, and, with the solution cooled in ice and vigorously stirred, the pH was carefully adjusted to 6·8 with dilute HCl. The supernatant was then precipitated at −5° by adding acetone to 35% (v/v), and as much precipitate (fraction III) as possible was removed by centrifuging at 5650 *g* in the MSE angle head (no. 69167) for 1 hr. Acetone was then added to the cloudy supernatant to a concentration of 45% (v/v) and the precipitate (fraction IV), which settled rapidly, was centrifuged. It was resuspended in water (120 ml.) either by hand or with a Vibromix (Shandon Scientific Co. Ltd., 63 Southern Row, London, W. 10), and the water suspension cooled in ice and stirred while being adjusted to pH 9·5–9·6 with 0·1 N-NaOH. After being stirred for 20–30 min. in ice, the solution was centrifuged for 30 min. at 90 000 *g*, and the clear supernatant removed (fraction V). The pH was adjusted to 7·4 with dilute HCl and an equal volume (usually 100 ml.) of 2 M-NaCl was slowly added. The pH of the solution sharply decreased by 0·5–1·0 unit on addition of the first few millilitres of NaCl and was brought back by the addition of alkali. Thereafter no further decrease occurred on the addition of NaCl to a final molar concentration. The solution was stirred at 2°

* Part 1: Heald (1959).

for 30 min. and centrifuged at 90 000*g* for 30 min. to yield a residue (fraction VI). This was dissolved in ice-cold water with the aid of dilute NaOH, care being taken to keep the pH below 9·5. After 30 min. stirring, solution was usually complete, the pH was adjusted to 7·4 and the whole solution was dialysed against distilled water (20–40 vol.) overnight at 2°. The solution was then centrifuged for 20 min. at 90 000*g* to remove traces of undissolved or precipitated material and the clear supernatant was freeze-dried to yield a pale-straw-coloured powder, yield 0·5–0·6 g./180 g. fresh wt. of brain. After freeze-drying, the material suffered some loss of solubility in water, the solutions being faintly turbid.

Lysolecithin. This was given by Dr C. Long.

Radioactive phosphorus. This was obtained as carrier-free orthophosphoric acid from The Radiochemical Centre, Amersham, Bucks.

Analyses

Total nitrogen. This was determined by distillation, after digestion as described by Chibnall, Rees & Williams (1943).

Total phosphorus. This was determined as orthophosphate after wet-ashing suitable samples of the material with perchloric acid (60%, w/v), as described by Long (1943).

Lipid phosphorus. Fractions in solution were first precipitated with trichloroacetic acid (to a concentration of 10%, w/v), and the residues extracted with 20 vol. of $CHCl_3$–methanol (2:1, v/v). The extracts were made up to volume and samples were taken for the determination of total phosphorus. Freeze-dried fractions were extracted for 2 hr. with ether–ethanol (1:3, v/v), and the extract was made to volume before sampling.

Cerebrosides. These were determined by a method of C. Long & D. A. Staples (unpublished work) with galactose as a standard.

Glycerol esters. These were determined by the method of Shapiro (1953) with tristearin as a standard.

Neuraminic acid. This was determined by the method of Long & Staples (1959) with synthetic *N*-acetylneuraminic acid (given by Dr C. Long, a gift to him from Miss P. Carroll) as a standard.

Nucleic acids. These were determined by the methods of Logan, Mannel & Rossiter (1952) in samples of the fractions after precipitation with trichloroacetic acid. Results are expressed as μmoles of P, with the extinction coefficient ϵ_P 9830.

Lipids. These were determined as the dry weight, at 110°, of lipid extracts made in $CHCl_3$–methanol (2:1, v/v) or in ethanol–ether (3:1, v/v).

Phosphoproteins. Phosphoprotein phosphorus and the radioactivity of phosphorylserine derived from acid hydrolysates of phosphoprotein were determined in fractions precipitated with trichloroacetic acid (10%, w/v) by methods described by Heald (1958). In freeze-dried preparations, phosphoprotein phosphorus was estimated by digesting samples in 2 ml. of N-NaOH at 37·5° overnight. The alkaline samples were acidified with 0·4 ml. of 10N-H_2SO_4 and after 30 min. in the refrigerator the residues were centrifuged. The entire supernatants were taken for the determination of orthophosphate.

Phosphorylation of phosphoprotein fractions. The phosphorylation of phosphoprotein fractions was examined in two systems: the liver mitochondrial system described by Burnett & Kennedy (1954) and a dispersion of whole guinea-pig cerebral cortex. Cortical tissue was dispersed in 5 vol. of 0·25M-sucrose and incubated in a medium containing, as final concentrations in 3·0 ml.: adenosine triphosphate, 2·5 mM; $MgCl_2$, 8 mM; sodium pyruvate, 13 mM; sodium fumarate, 1·6 mM: KCl, 50 mM; diphosphopyridine nucleotide, 0·5 mM; cytochrome *c*, 8·3 μM; sodium phosphate buffer, pH 7·4, 0·01M; 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer, pH 7·4, 50 mM; together with radioactive phosphate and the fraction dissolved in water. Incubations were at 37·5° for 30 min. in air.

RESULTS

Distribution of phosphoprotein in dispersions of guinea-pig brain. Previous work demonstrating the presence of a phosphoprotein attached to particulate matter had been carried out largely with tissue slices incubated in saline media (Heald, 1959). Under these conditions the greater part of such phosphoprotein was attached to particles sedimented at 1500*g* for 10 min., i.e. in the 'nuclear' fraction. This distribution also holds for particles centrifuged from dispersions of guinea-pig cerebral cortex prepared immediately after removal of the brain from the animal. Table 1 shows that the nuclear fraction contains the greater quantity

Table 1. *Distribution of phosphoproteins in subcellular fractions from guinea-pig brain*

Tissues were fractionated by centrifuging dispersions made in 0·25M-sucrose (containing 0·5 mM-EDTA), according to the method of Brody & Bain (1952). Total phosphoprotein and the radioactivity of phosphorylserine released from phosphoprotein upon acid hydrolysis were determined as described in the text.

		Fraction		
		Nuclear	Mitochondria + microsomes	Supernatant
Phosphoprotein phosphorus	(μmoles of P/g. wet wt.)	0·41	0·21	0·90
	(% of total)	27·0	13·8	59·2
Nitrogen (mg./g.)		6·7	8·3	10·2
Phosphoprotein phosphorus	(μmoles of P/g. wet wt.)	0·37	0·20	0·90
	(% of total)	25·0	13·6	61·0
Nitrogen (mg./g.)		6·1	8·9	10·7
Radioactivity of phosphorylserine	(counts/min.)	1312	438	2432
	(% of total)	31·4	10·5	58·1

of particulate phosphoprotein, and that it is present in a higher concentration, in terms of nitrogen, than in the mitochondrial fraction. Also in Table 1 are presented the results of an experiment in which the phosphoproteins were made radioactive by injecting radioactive orthophosphate intracisternally into a guinea pig. After 3 hr. the brain was removed and the particulate fractions were isolated. The distribution of radioactivity in phosphorylserine derived from the phosphoprotein closely paralleled that of phosphoprotein phosphorus determined by direct analysis. The larger quantity of phosphoprotein in the supernatant fraction of such dispersions is similar to that found previously (Heald, 1959). This fraction has been shown to be metabolically distinct from phosphoproteins attached to particles. Since the greatest concentration of phosphoprotein attached to particulate matter was found in the nuclear fraction, this fraction was chosen as the starting material.

A characteristic feature of the phosphoprotein sought is the ability to yield phosphorylserine on acid hydrolysis. To enable this to be detected more readily, the phosphorus of the phosphoprotein was labelled with radioactive phosphate by incubating cerebral slices with radioactive phosphorus in the presence of glucose. Particulate fractions, prepared from these slices, were added to those prepared from ox brain. After hydrolysis of samples of the appropriate fractions, phosphorylserine was separated chromatographically and the total radioactivity determined. A similar use of radioactive phosphorylserine has been made by Glomset (1956, 1957) during the fractionation of phosphoproteins from the cytoplasm of rat liver. As the number of fractions increased, the radioactivity decayed or was reduced by loss to an extent where such measurements were no longer reliable, and accordingly measurements of phosphoprotein were also made in terms of alkali-labile phosphorus.

Solubilization of phosphoproteins from particles. Particulate fractions were extracted in a variety of ways, some of which are listed in Table 2. Of those tested, only Teepol L was effective in solubilizing the phosphoprotein without loss. The use of saline media at pH 8·0 (Expts. 1 and 2), conditions found to be optimum for the extraction of cerebral proteins (LeBaron & Folch, 1959), was ineffective, as was the use of butan-1-ol (Morton, 1955). Other conditions tested but found either to result in a total loss of radioactivity or to fail to extract phosphoprotein were: extraction with aqueous buffers (sodium acetate, 0·2 M, pH 5·0, and tris–hydrochloric acid, 0·5 M, pH 8·6), either of the fresh particulate material or after dehydrating in 10 vol. of acetone; extraction of the acetone-dried material with butan-1-ol either by the one- or two-

Table 2. *Effects of various extraction conditions upon the solubilization of phosphoprotein from particulate fractions of brain*

The particulate fractions were prepared as described in the Materials and Methods section. Radioactivity of phosphoprotein determined as phosphorylserine was estimated after acid hydrolysis of samples treated as described in the text. Extractions were performed by stirring for 0·5–2·0 hr. at the temperature indicated.

Extractant	Extraction		Particles before extraction		Extract		Residue	
	Time (hr.)	Temp.	Phosphoryl-serine (counts/min.)	N or protein (mg.)	Phosphoryl-serine (counts/min.)	N or protein (mg.)	Phosphoryl-serine (counts/min.)	N or protein (mg.)
1. NaCl (*I* 2·5 in 0·1 M-tris–HCl, pH 8·0)	2	2°	10 200	252*	1177	69·0*	3260	174*
2. $MgSO_4$ (*I* 1·6 in 0·1 M-tris–HCl, pH 8·0)	2	2	5 500	268*	734	154·0*	4762	114·0*
3. Butan-1-ol, 1 phase (pH 7·0) (Morton, 1955)	1·5	2	3 805	15·0	209	1·9	—	—
4. Sodium deoxycholate (1 %, w/v)	0·5	17–20	1 301	2·26	10	1·44	338	0·80
5. Lysolecithin (4 mM)	0·5	17–20	600	3·4	137	2·14	0·0	1·33
6. Teepol L (0·2 ml./g. of tissue)	0·5	17–20	4 291	3·3	4293	2·06	0·0	0·8
7. Teepol L (0·2 ml./g. of tissue)	0·5	17–20	1 054	2·7	1046	1·4	0·0	1·32

* Protein estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

phase procedures at pH 7·4–8·6 (with tris–hydrochloric acid, 0·03 M, as buffer). Although extraction with magnesium sulphate (Table 2) yielded a residue containing a greater amount of radioactive phosphoprotein (in terms of nitrogen) than the original particles, the residual material was not solubilized by Teepol L. Cetavlon (cetyltrimethylammonium bromide) yielded results similar to those obtained with lysolecithin.

Preparation of partially purified phosphoprotein. The conditions chosen to yield a partially purified preparation still retaining radioactivity in phosphorylserine are described in detail in the Materials and Methods section. Steps in the procedure are given in Table 3 showing a typical preparation. In terms of total phosphorus the preparation was purified some 15 times over the original starting material with a yield of about 25% as judged by radioactivity remaining in the phosphorylserine. This figure agrees with recoveries of alkali-labile phosphorus. Thus in two other preparations from 180 g. of ox brain the quantities of alkali-labile phosphorus were: fraction I, 43 and 44 μmoles of P; fraction VII, 12·0 and 14·3 μmoles of P. Purification in terms of nitrogen was fourfold.

Certain points may be noted in connexion with the individual steps. The nuclear fraction used as the starting material contains small amounts of a phosphoprotein phosphatase (P. J. Heald, unpublished work) the pH optimum of which is 5·5–6·0. Little activity is shown above pH 7·0. A simple and complete inhibitor for this enzyme has not yet been found. The enzyme is considered to be responsible for the loss of phosphoprotein in the stages before acetone precipitation. Since no precipitate could be obtained with acetone (to 50%, v/v) above pH 7·0, precipitations were performed rapidly at pH 6·8. No phosphatase was detectable in the precipitates. The phosphoprotein is not stable in dilute alkali. Thus in one experiment after incubation of fraction VII at 37·5° for 30 min. at different pH values the recoveries of radioactive phosphorylserine derived from the phosphoprotein were: pH 5·6, 73·5%; pH 7·7, 27%; pH 9·2, 10%. The lability is thus greater than that of the radioactive phosphorus in phosphorylated casein (Sundararajan, Kumar & Sarma, 1960). For these reasons it was necessary to carry out the fractionations as rapidly as possible without exposing the extracts to strongly alkaline conditions for too long.

Precipitation of fraction VII from fraction V was best achieved with M-NaCl, pH 7·4, which removed considerable quantities of nucleic acid, phosphorus and nitrogen. In addition to the results in Table 3 three other experiments showed that only 3–9% of the nucleic acid present in fraction V was found in the precipitate. Ammonium

Table 3. *Steps in the isolation of a phosphoprotein fraction from cerebral particles*

For details see Materials and Methods section.

Fraction	Vol. (ml.)	Nitrogen (mg.)	Total P (μmoles)	Lipid P (μmoles)	Nucleic acid P (μmoles)	Radioactivity in phosphorylserine from phosphoprotein (counts/min.)	Counts/min./mg. of N	Counts/min./μmole of P
I. Total particles	355	935	9200	3700	246	10 000	11·2	1·1
II. Teepol extract	225	415	1480	745	105	6 350	15·3	4·3
III. 0–36% Acetone	33	46	320	—	11·5	680	14·8	2·1
IV. 36–45% Acetone	150	242	890	508	60·0	4 700	19·5	5·3
V. 36–45% Acetone (alkali-sol.)	138	152	610	250	44·0	4 700	31·0	7·7
VI. 36–45% Acetone (alkali-insol.)	40	—	280	—	—	450	—	—
VII. NaCl insol.	95	69	186	140	11·6	2 740	40·0	14·8

sulphate (I 0·1, pH 7·4) or precipitation at pH 5·0 failed to yield any purification, the entire material being precipitated. Attempts to remove lipids by treatment with excess of butan-1-ol (cf. Sundararajan *et al.* 1960) over a pH range 6·5–8·6 were unsuccessful since the entire radioactive protein collected as a denatured layer at the interface. Virtually no protein passed into solution. Fraction V was not absorbed on carboxymethylcellulose, pH 5·0, but was firmly retained by diethylaminoethylcellulose at pH 7·4. From this material it could be eluted only by alkaline buffers and 0·1 N-sodium hydroxide. No radioactive phosphorylserine was obtained from any fraction, probably owing to the alkali lability of the phosphorus.

The gross composition of three preparations is given in Table 4. As will be seen, the quantity of lipids in preparation 1 was higher than in the other preparations and the total quantity of phosphorus in the lipid-free residues was lower. Of this phosphorus 50–80 % was labile in alkali. Lipids were extracted by shaking with ethanol–ether (3:1, v/v) for 2 hr. at 2°, after which time no further quantities were removed. The amount removed was similar to that extracted from moistened samples with chloroform–methanol (2:1, v/v) and was not increased by extracting with acidified ethanol–ether. No material was extracted by dry chloroform–methanol, indicating that free lipid was absent. The Lieberman–Burchard reaction was negative. Glycerol esters in the lipid extracts, determined by the method of Shapiro (1953) with tristearin as a standard, yielded values calculated as tristearin almost equivalent to the dry weight of the lipids. Since the extracts contain little cerebroside and about 25–30 % of phospholipids (P × 25) it is considered that the remaining lipids estimated by the hydroxamate method consist largely of triglycerides. The lipid-free residue contained 11·2–12·0 % of nitrogen, a value similar to that found by Folch & Lees (1951) for the protein of ox-brain proteolipids. However, in contrast with the latter material, the percentage of nitrogen in the phosphoprotein was not increased on boiling with chloroform–methanol (2:1, v/v) for 6 hr. Hexosamines were not detectable and neuraminic acid was absent. Examination for carbohydrates released on acid hydrolysis in N-hydrochloric acid for 3 hr. (Heald, 1951) was negative. Qualitative examination of the amino acids revealed a preponderance of acidic amino acids (Table 5) together with appreciable quantities of serine. In the absence of a quantitative estimation of the individual acids present it was not possible to decide whether the low nitrogen values were in fact due to raised quantities of glutamic and aspartic acid, or indicated lipid material firmly bound to the protein. After removal of the lipids

Table 4. *Composition of phosphoprotein fractions isolated from cerebral particles*

For preparation and analytical methods see text.

Prep. no.	Total P (%)	Total N (%)	Lipids (% by wt.)	Lipid-free residue				Lipid extract					Glycerol esters calculated as tristearin (%)
				N (%)	P (%)	Alkali-labile P (%)	Hexosamine (%)	N (%)	P (%)	'Galactose' (%)	Neuraminic acid (%)		
1	0·31	5·0	66·0	11·2	0·1	0·05	0	—	—	—	—		—
2	0·65	8·6	43·0	11·6	0·21	0·10	0	2·0	0·96	2·60	—		85·0
3	0·44	8·1	38·0	12·0	0·24	0·20	0	1·16	1·17	1·18	0·31		108·0

by ether–ethanol, the residues were insoluble in water but dissolved slowly in alkali.

Phosphorylation of the fractions. A primary purpose of the study described above was to obtain preparations for use in the study of phosphorylation and dephosphorylation systems. The results are presented in Table 6. In Expts. 2 and 3, phosphoprotein phosphorylation was detected by the increased amount of radioactive phosphorylserine found in the hydrolysed phosphoprotein at the end of the experiments. In Expt. 1, the phosphoprotein fraction, after phosphorylation, was isolated as described by Burnett & Kennedy (1954) and counted directly. In all three experiments it was clear that the fractions obtained were capable of accepting radioactive phosphate, which appeared as phosphorylserine on acid hydrolysis of the phosphoprotein.

Attempts at further fractionation of fraction VII. A preliminary examination of fraction V in the analytical ultracentrifuge was made by Dr K. V. Shooter, using an aqueous 0·5% solution at pH 8·0–9·0. Only one peak was observed, the peak being asymmetric (S $0{\cdot}39 \times 10^{-13}$). However, the area under the peak was small, suggesting that some material could have been present as large aggregates easily spun down. Since no boundary was seen during acceleration any such aggregates must have been very polydisperse in size. Examination by electrophoresis on paper or in a starch gel showed that the bulk of the material remained firmly attached to the starting line on the paper and did not enter the gel. Precipitation curves obtained in sodium chloride solutions (Fig. 1)

Table 5. *Amino acids in phosphoprotein fractions isolated from brain*

Protein was hydrolysed for 12 hr. in 6N-HCl. After removal of the HCl under vacuum the hydrolysate, in water, was spotted on paper and run first in butanol–acetic acid–water (120:30:50, by vol.) and secondly in phenol–water (160:40, w/w).

Amino acid	Relative intensity
Alanine	+ + + +
Arginine	+ +
Aspartic acid	+ + +
Cysteine	+ (weak)
Glutamic acid	+ + + +
Glycine	+ +
Histidine	+
Lysine	+ +
Phenylalanine	+
Proline	+
Serine	+ + +
Threonine	+ +
Tyrosine	+
Valine	+ +
(Leucine) (*iso*Leucine)	+ + +

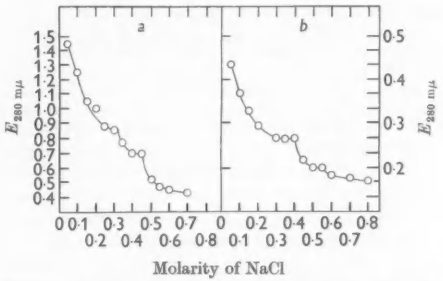

Fig. 1. Precipitation of components of crude brain phosphoprotein by increasing quantities of NaCl. Buffer: tris, pH 7·4, I 0·05. *a*, Protein concentration 3·35 mg./ml.; *b*, protein concentration 1·0 mg./ml. Equilibrium 18 hr. at 2°. All solutions were centrifuged for 7 min. at 100 000 *g* before $E_{280\ m\mu}$ was read.

Table 6. *Phosphorylation of brain phosphoprotein fractions*

In Expt. 1 the mitochondrial system of Burnett & Kennedy (1954) was used. In Expts. 2 and 3 a fortified cerebral dispersion was used (see text). In Expt. 1 the counts were obtained from the total phosphorus present in the protein residues prepared as described by Burnett & Kennedy (1954). In Expts. 2 and 3 the counts are counts in phosphorylserine separated after acid hydrolysis of the fractions.

Expt. no.	System	Counts/min. in system	O_2 uptake (μl.)	^{32}P added (μc)
1	Mitochondria	8 500	44·0	10·0
	Mitochondria + phosphoprotein	20 800	47·0	
	Phosphoprotein	260	0·0	
2	Dispersion	321	—	2·0
	Dispersion + phosphoprotein	480	—	
	Phosphoprotein	0	—	
3	Dispersion	216	248·0	2·0
	Dispersion + phosphoprotein	594	290·0	
	Phosphoprotein	0	0·0	

Table 7. *Analysis of phosphoprotein fractions prepared by subfractionation of fraction VII in sodium chloride solutions*

Conditions are as in Fig. 1 (*a*)

				Lipid-free residue			Lipid extract				
Fraction	Total P (%)	Total N (%)	Lipids (%)	N (%)	P (%)	Alkali-labile P (%)	N (%)	P (%)	Galactose (%)	Neuraminic acid (%)	Glycerol esters calculated as tristearin (%)
(A)	0·50	7·75	40·5	10·9	0·18	0·1	1·20	1·15	0·96	0·38	115·0
(B)	0·20	6·8	31·0	12·0	0·15	0·05	3·30	0·45	1·70	—	85·0

revealed the presence of at least three protein-containing substances. Quantities of the two major fractions were prepared under the conditions described for *a* in Fig. 1 and were analysed. The results are given in Table 7. Apart from the differences in solubility, the composition of the lipid extracts were also different. No quantitatively detectable differences were noted in the amino acid pattern from hydrolysed samples of the lipid-free residues, which were similar to that shown in Table 5.

DISCUSSION

As stated at the outset an object of this work was to obtain from a preparation of brain particulate material a soluble fraction containing phosphoprotein for use as a substrate in further studies upon phosphorylation and dephosphorylation by cerebral enzymes. It has been found possible to prepare such a fraction by extraction of the subcellular particles with a detergent. The fraction is a lipophosphoprotein complex and has been purified 10–14 times in terms of phosphorus and 4–5 times in terms of nitrogen. The preparation obtained cannot be considered to be a single entity, for examination of the precipitation curves obtained with increasing concentrations of sodium chloride showed that at least three components were present, the major ones differing in gross composition. Whether these differences do indeed indicate the presence of two or more phosphoproteins or whether they represent a similar protein combined to a differing extent with different lipids cannot be assessed from the present data and requires further study by other techniques. The high proportion of glycerol esters (possibly triglyceride in nature) and the absence of cholesterol are of considerable interest since all glycerol-containing lipids so far characterized in brain are phospholipids (LeBaron & Folch, 1957). The precise nature of these esters remains to be established. The absence of cholesterol could conceivably be due to a dissociation of a lipoprotein–cholesterol complex by the anionic detergent used in the extraction (Rideal & Taylor, 1957). However, it has been found (P. J. Heald & M. H. Cohen, unpublished work), in a more extended study of the chemical and morphological characteristics of the subcellular fractions containing the phosphoprotein, that the fraction richest in phosphoprotein is amongst the poorest in cholesterol. The material is therefore not associated with myelin. Since it has been shown that the phosphoprotein is also not associated with nuclei, mitochondria or microsomes (Heald, 1959) it seems possible that the material is a constituent of the cell walls, in which triglycerides have been considered to be essential components (see Burgen, 1957).

There is little previous work upon the nature of phosphoproteins of animal tissues, other than enzymes listed previously (Heald, 1959). Glomset (1957) showed that the separation of rat-liver cytoplasmic proteins, obtained by dispersing liver in aqueous sucrose solutions, upon columns of diethylaminoethylcellulose, yielded several fractions containing phosphorylserine but the identity of these was not further investigated. More recently, Sundararajan *et al.* (1960) obtained a soluble fraction, containing phosphoprotein, from rabbit mammary gland by extraction with dilute alkali at pH 9·7 and removal of lipids with butan-1-ol. It was not clear whether this preparation was contaminated with casein. Both these preparations differ from that now described, which is not readily eluted from diethylaminoethylcellulose and is degraded by treatment with dilute alkali or butan-1-ol. Though the quantities of alkali-labile phosphorus are somewhat lower than those found in phosphoprotein fractions obtained from milk (Perlman, 1955), egg yolk (Fevold, 1951; Joubert & Cook, 1958) or the plasma of the laying hen (McIndoe, 1959), this is considered to be due to dephosphorylation occurring during the initial stages of purification. Cerebral tissues contain an active phosphoprotein phosphatase (Feinstein & Volk, 1949), which probably contributes to the major loss of phosphate which occurred during the preparation.

A significant feature of the fractions obtained is their retention of the ability to become phosphoryl-

ated when incubated in suitable phosphorylating systems. It has been convincingly demonstrated (Rabinowitz & Lipmann, 1958, 1960; Burnett & Kennedy, 1954) that of several purified proteins containing phosphorus only a few, including casein and phosvitin, or peptones derived from these substrates, are capable of acting as phosphate acceptors when incubated with mitochondria or with a purified phosphokinase. Such a specificity is likely to be due to the possession of a specific configuration which, with casein and phosvitin, can be destroyed by prolonged treatment with dilute alkali (Burnett & Kennedy, 1954; Rabinowitz & Lipmann, 1960). Thus the finding that cerebral phosphoprotein is also capable of accepting phosphate in a similar phosphorylating system indicates that this degree of structural specificity has been retained throughout the isolation procedure. As with casein (Burnett & Kennedy, 1954, see also Table 6), only radioactive phosphorylserine could be detected in hydrolysates of the phosphoprotein fraction. It has been well established that serine itself is not phosphorylated by liver mitochondria or by purified phosphokinase, and discussions of the manner in which serine in phosphoproteins becomes radioactive are commonly made in terms of the structures surrounding the active serine residue in trypsin and chymotrypsin (cf. Sundararajan *et al.* 1960). However, a general reactivity of a serine site in an enzyme does not make it capable of accepting phosphate via a protein phosphokinase reaction, for trypsin, chymotrypsin, pepsin, hexokinase, ribonuclease and muscle phosphorylase *b* are not phosphorylated by liver mitochondria (Burnett & Kennedy, 1954) or by purified yeast protein phosphokinase (Rabinowitz & Lipmann, 1960). On this basis the phosphoprotein fraction from brain might be expected to possess some structural similarities to casein and phosvitin, in which the sequences of linked phosphorylserine residues found may be considered as possible sites for phosphorylation (Williams & Sanger, 1959).

SUMMARY

1. A lipophosphoprotein fraction has been prepared from easily sedimenting particles of ox brain. The preparation contains 8% of nitrogen, 0·3–0·6% of P and 40–66% of lipids. Calculated in terms of the total phosphorus content of the particles, the preparation has been purified 10–14 times. Calculated in terms of the total nitrogen content of the particles it has been purified 4–5 times. The recovery is 25% of the starting material. The preparations are not pure and can be separated into at least two major components by salt fractionation.

2. The lipid-free residue contains 11·2–12·0% of nitrogen. This figure is not increased by exhaustive extraction of the residue. It contains 0·1–0·24% of phosphorus of which 50–75% is labile in alkali. Qualitative analysis revealed an amino acid pattern suggestive of an acidic protein.

3. The fraction does not contain cholesterol. Neuraminic acid and galactose from cerebrosides are present only in small amounts. The lipids contain a high proportion of glycerol esters thought to be triglycerides.

4. When incubated either with a fortified cerebral dispersion or with rat-liver mitochondria in the presence of radioactive phosphate, the preparations are phosphorylated, radioactive phosphorylserine being detected in hydrolysates of the phosphoprotein at the end of incubation.

It is a pleasure to thank Dr C. Long for a gift of *N*-acetylneuraminic acid and for describing the method for determination of cerebrosides before publication. Thanks are also due to Dr K. V. Shooter, for ultracentrifuging various preparations.

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Studies on Pituitary Adrenocorticotrophin

3. IDENTIFICATION OF THE OXIDATION–REDUCTION CENTRE*

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The oxidation–reduction behaviour of corticotrophin A_1 was studied in detail by Dedman, Farmer & Morris (1957). The hormone is oxidized by hydrogen peroxide under appropriate conditions to give a biologically inactive product which can be distinguished chromatographically from the untreated corticotrophin (Dixon & Stack-Dunne, 1955; Farmer & Morris, 1956). Reduction of the oxidized hormone with thiol compounds completely restores the biological activity, and the reduction product is chromatographically identical with the original untreated hormone. The oxidation–reduction process is thus readily reversible.

Dedman *et al.* (1957) examined the potentially oxidizable amino acid residues in corticotrophin A_1 and concluded that it was difficult to explain the experimental results on the basis of the known structure. In particular, methionine was excluded because Raney-nickel-treated corticotrophin, which was apparently sulphur-free, exhibited the characteristic oxidation–reduction behaviour. The subsequent inability to isolate α-aminobutyric acid from Raney-nickel-treated corticotrophin A_1 has prompted a re-examination of this material. Improved analytical methods with hydrolysis by leucine aminopeptidase show that it contains methionine, which was destroyed by the earlier method of acid hydrolysis. The evidence excluding this amino acid from participation in the oxidation–reduction centre of corticotrophin is in fact the thioether grouping of methionine.

* Part 2: Dedman, Farmer & Morris (1957).

EXPERIMENTAL

Bioassay methods. The biological activities of all preparations were measured by the adrenal-ascorbic acid-depletion method of Sayers, Sayers & Woodbury (1948) with minor modifications (Morris, 1951).

Preparation of corticotrophin A_1. The hormone was isolated from pig anterior-pituitary glands by the method of Dedman *et al.* (1957), or by the method of Farmer (1959). The oxidized hormone was prepared by H_2O_2 oxidation as described by the former authors.

Preparation of leucine aminopeptidase. The enzyme was prepared according to the procedure of Hill & Smith (1957). Enzymic activity was measured by the digestion of leucyltyrosine. The enzyme was stored at 2° as a solution in 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 8·0, which was 0·05 M with respect to $MgCl_2$. The enzyme was activated before use by heating for 30 min. at 40° at pH 8·5 (Hill & Smith, 1957).

Preparation of the tryptic-digestion products of corticotrophin A_1 *and oxidized corticotrophin* A_1. (*a*) Digestion. Corticophin A_1 (9·8 mg.) was dissolved in an aqueous 0·025% solution of trypsin (L. Light and Co. Ltd., 50 Northrop units/mg.) in 0·01 M-sodium phosphate buffer, pH 8·0 (1·0 ml.). The mixture was maintained at 30° for 30 min. 1 M-Pyridine–acetate buffer (pyridine, 16 ml.; 2 N-acetic acid, 100 ml.), pH 4·4 (0·11 ml.), was added and the mixture was heated at 90° for 2 min. to inactivate the trypsin. Oxidized corticotrophin A_1 (8·8 mg.) was also digested by this procedure.

(*b*) Chromatography. Carboxymethylcellulose (0·6 m-equiv./g.; 2 g.) was suspended in aqueous 0·5% pyridine and gently dispersed in an all-glass Potter–Elvehjem homogenizer. After the greater part of the material had sedimented, the supernatant liquid and very fine material were removed. The carboxymethylcellulose was washed several times with 0·067 M-pyridine–acetate buffer, pH 4·4 (redistilled pyridine, 5·30 ml.; 2 N-acetic acid, 66·7 ml.; diluted to 1 l. with water). A column 62 cm. × 0·5 cm. was packed

with the carboxymethylcellulose in approx. 10 cm. sections. The tryptic hydrolysate was applied to the top of the column and development of the chromatogram carried out with 0·067 M-pyridine–acetate buffer, pH 4·4, at a flow rate of 5 ml./hr. Usually 100 × 1·55 ml. fractions were collected. Fig. 1 (column 1) shows the separation of the tryptic-digest peptides from corticotrophin A_1, and Fig. 1 (column 2) shows the corresponding separation from oxidized corticotrophin A_1.

Zone A (Fig. 1, column 1) is a mixture of the *C*-terminal peptides of corticotrophin A_1 and corresponds mainly to the peptide T_1 obtained by Shepherd *et al.* (1956) from a tryptic digest of β-corticotrophin. Zone B is the *N*-terminal peptide (T 10 of Shepherd *et al.* 1956), containing residues 1–8 of the corticotrophin molecule, and zone C is the adjacent peptide (T 14 of Shepherd *et al.* 1956), comprising residues 9–15. The very basic peptide T 17 of Shepherd *et al.* (1956) remained on the column during the development and could only be recovered by alkaline elution. The chromatographic separation of the tryptic-digestion products of corticotrophin A_1 reported here with a 62 cm. column is markedly superior to that obtained after countercurrent distribution through 1400 transfers by Shepherd *et al.* (1956). Zones A′, B′ and C′ are the corresponding peptides from oxidized corticotrophin A_1. In several experiments zone B′ moved slightly faster than zone B.

The peptides could be recovered from the appropriate pooled fractions by direct freeze-drying.

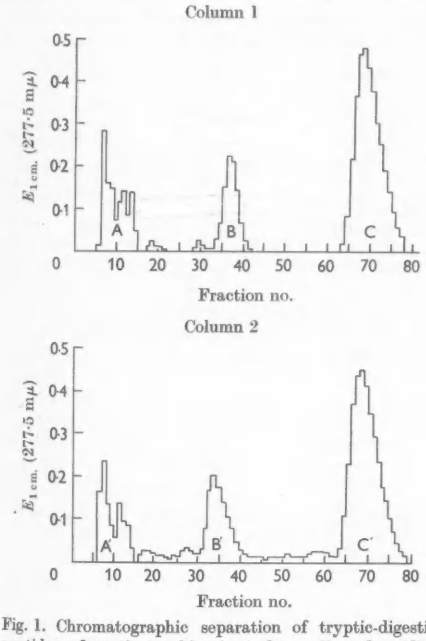

Fig. 1. Chromatographic separation of tryptic-digestion peptides of corticotrophin A_1 (column 1) and oxidized corticotrophin A_1 (column 2) on carboxymethylcellulose in 0·067 M-pyridine–acetate buffer, pH 4·4. For designations of individual peptides see text.

Chromatographic comparison of the T 10 *and* T 14 *peptides from corticotrophin* A_1 *and oxidized corticotrophin* A_1. A 127 cm. × 0·6 cm. column was prepared from 6 g. of carboxymethylcellulose in the 0·067 M-pyridine–acetate buffer. A mixture of peptide B (1·0 mg.) and peptide B′ (1·25 mg.) was applied to the column and development continued with the pyridine–acetate buffer. Fig. 2 (column 3) shows the course of the separation.

A similar experiment was carried out with peptides C (0·75 mg.) and C′ (0·75 mg.) and the corresponding chromatogram is shown in Fig. 2 (column 4).

Acid hydrolyses of peptides. Hydrolyses were carried out in a sealed tube with constant-boiling-point HCl (0·25 ml.) for periods of 24–48 hr.

Leucine aminopeptidase hydrolyses. In representative experiments, 0·1–1·0 mg. of the material to be hydrolysed was dissolved in 0·2–0·5 ml. of water and 0·2–0·5 ml. of a solution of activated leucine aminopeptidase was added. The mixture was kept at 40° for 18 hr., and the reaction was terminated by the addition of 2 N-acetic acid (0·2 ml.). The digestion mixture was evaporated to dryness *in vacuo*. For paper-electrophoretic analysis the residue was dissolved in 10 μl. of formic acid–acetic acid solution (see below) and a 5 μl. portion was taken for analysis. Paper-

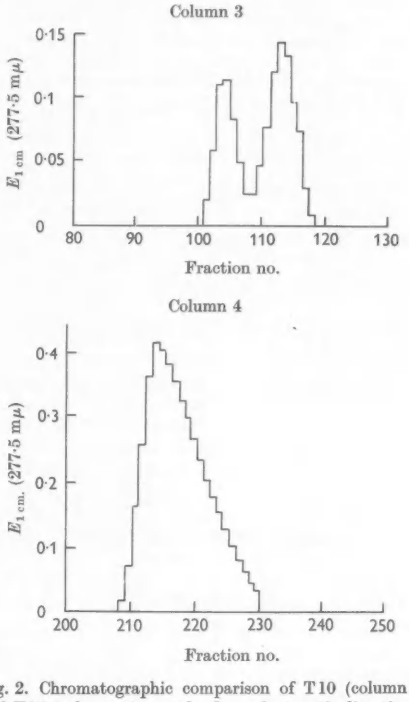

Fig. 2. Chromatographic comparison of T 10 (column 3) and T 14 (column 4) peptides from the tryptic digestion of untreated (B and C) and of oxidized (B′ and C′) corticotrophin A_1 on carboxymethylcellulose in 0·067 M-pyridine–acetate buffer, pH 4·4.

chromatographic examination of the products of prolonged autodigestion of the enzyme showed only a very small amount of glutamic acid.

Amino acid analyses. In the earlier part of this work amino acid separations were carried out by paper chromatography in the system butan-1-ol–acetic acid–water (60:15:25, by vol.). The amino acid spots were developed with ninhydrin according to the method of de Wael & Cadavieco (1954) and the pigment was eluted with aqueous 50% (v/v) propan-1-ol. Extinction values were determined at 570 mμ. Standard quantities of all amino acids measured were chromatographed and developed simultaneously.

Later analyses were carried out by high-voltage electrophoresis on paper by a simplified form of the method of Atfield & Morris (1960). The separation was carried out on a 65 cm. × 15 cm. strip of Whatman no. 3 MM paper in the apparatus of Gross (1956). A solution containing formic acid (40 g.) and acetic acid (155·4 g.) in water (2 l.), pH 1·9, was used as electrolyte for the majority of the separation of neutral and acidic amino acids. This system could be used for the determination of glycine, serine, tyrosine, methionine, methionine sulphoxide, glutamic acid and phenylalanine by electrophoresis at 25 v/cm. for 7 hr. Determination of the basic amino acids histidine, arginine and lysine was carried out in pyridine–acetate buffer, pH 6·1 (5% pyridine in aqueous 0·5% acetic acid, v/v), at 25 v/cm. for 3·5 hr. The amino acids were estimated after separation by the cadmium–ninhydrin method of Heilmann, Barollier & Watzke (1957), the extinction of the red complex being measured at 500 mμ.

RESULTS

Amino acid composition of T10 *peptides from corticotrophin* A_1 *and oxidized corticotrophin* A_1. The analyses carried out by quantitative paper chromatography on a HCl hydrolysate of the chromatographically separated peptides are given in Table 1. Methionine sulphoxide was detected qualitatively in a preliminary paper-electrophoretic examination of the oxidized hormone. It was absent from the untreated material.

Leucine aminopeptidase hydrolyses of T10 *peptides from corticotrophin* A_1 *and oxidized corticotrophin* A_1. Preliminary qualitative experiments in which the preparations were digested with leucine aminopeptidase followed by high-voltage electrophoretic separation of the liberated amino acids showed that the T10 peptide (B) from corticotrophin A_1 contained serine, tyrosine, methionine,

Table 1. *Amino acid composition of* T10 *peptides from untreated and oxidized corticotrophins*

Hydrolysis was with HCl.

	Corticotrophin A_1	Oxidized corticotrophin A_1
	(moles of amino acid/mole of peptide)	
Serine	1·56	1·60
Tyrosine	0·42	0·43
Methionine	0·58	0·09
Glutamic acid	1·33	0·97
Histidine	0·82	0·72
Phenylalanine	0·89	0·81
Arginine	0·97	0·97

Table 2. *Amino acid contents of corticotrophin* A_1 *and its Raney-nickel reaction product*

Hydrolysis was with leucine aminopeptidase.

	Corticotrophin A_1	Reaction product (mean of two determinations)
	(moles of amino acid/mole of corticotrophin)	
Serine	2·00	2·00
Methionine	0·51	0·82
Methionine sulphoxide	0·0	0·0
Glutamic acid	0·98	0·89
α-Aminobutyric acid	—	0·00

Table 3. *Biological activities and amino acid analysis of oxidized and untreated corticotrophin*

Potencies are means with fiducial limits.

Preparation	Biological activity (relative to untreated hormone as unity)	Amino acid content (moles/mole of peptide)			
		Serine	Methionine	Methionine sulphoxide	Glutamic acid
A_1 (1)	1	2·00	0·51	0·0	0·98
A_1 (2)	1	2·00	0·93	0·08	1·03
A_1 (3) (rechromatographed)	1	2·00	0·94	0·00	1·04
A_1 (4)	1	2·00	0·94	0·07	0·93
Oxidized A_1 (1)	0·02	2·00	0·0	0·89	0·80
Oxidized A_1 (2)	0·024 (0·019–0·032)	2·00	0·05	0·82	1·00
Partially oxidized A_1 (3)	0·60 (0·47–0·77)	2·00	0·58	0·40	0·86
Partially oxidized A_1 (4)	0·93 (0·72–1·21)	2·00	1·00	0·16	0·74

glutamic acid, phenylalanine, histidine and arginine, in accordance with its established structure. The corresponding peptide (B′) from oxidized corticotrophin A_1 contained serine, tyrosine, methionine sulphoxide, glutamic acid, phenylalanine, histidine, arginine.

Amino acid analysis on untreated and oxidized corticotrophin A_1 and the Raney-nickel reaction products after hydrolysis with leucine aminopeptidase. The materials were hydrolysed with leucine aminopeptidase and the products subjected to paper-electrophoretic separation and quantitative colorimetry as described in the Experimental section. Results are given as mole fractions of amino acid referred to 2 moles of serine as standard.

The amounts of certain amino acids in the *N*-terminal sequence of Raney-nickel-treated corticotrophin A_1 are compared with those in the starting material in Table 2. The analytical method was capable of detecting α-aminobutyric acid down to a mole fraction of 0·1 on the amounts hydrolysed.

A HCl hydrolysate of the Raney-nickel-treated material had a mole fraction of methionine of 0·28.

Values for untreated, partially and completely oxidized preparations of corticotrophin are given in Table 3.

DISCUSSION

The chromatographic separation of the T10 peptides from tryptic digests of oxidized and untreated corticotrophins and the apparent identity of the T14 peptides makes it probable that the oxidation–reduction centre is located in the *N*-terminal sequence of eight amino acids. The T1 and T17 peptides from this digest were not compared directly but the absence of oxidizable residues in this portion of the molecule makes the location of the oxidation–reduction centre there unlikely. The complete hydrolysis of the T10 peptide from oxidized corticotrophin by leucine aminopeptidase makes it improbable that this sequence contains any unusual residue, and also establishes the optical configuration of the amino acids in this sequence.

The only significant difference in compositions between the T10 peptides of the corticotrophins in the two cases lies in the conversion of methionine into methionine sulphoxide in the oxidized form (Tables 1 and 3).

Dedman *et al.* (1957) had considered participation of the thioether group of methionine in the oxidation–reduction centre to be unlikely on account of the normal oxidation–reduction behaviour of corticotrophin in which this group had apparently been removed by treatment with Raney nickel under vigorous conditions. Hydrolysis of this material by leucine aminopeptidase shows, however, that it still contains methonine (Table 2) but no α-aminobutyric acid. Acid hydrolysis, however, confirms the earlier experiments in showing a greatly diminished yield of methionine. The loss is presumably due to destruction in the presence of trace amounts of nickel in the preparations, despite ion-exchange purification. Hayes & White (1954) have previously observed the peculiar lability of methionine in the corticotrophin molecule.

The presence of methionine and the absence of α-aminobutyric acid in the Raney-nickel-treated preparation thus removes the objections to methionine as the oxidizable residue in the corticotrophin molecule, and the apparent correlation between the biological activity and the methionine content shown in Table 3 provides evidence that the thioether group is in fact the oxidation–reduction centre. The chemical reactivity of this centre described in the preceding paper of this series is also in agreement with this view.

SUMMARY

1. The products of tryptic digestion of corticotrophin A_1 and oxidized corticotrophin have been separated and compared by chromatography on carboxymethylcellulose. Only the *N*-terminal peptides differed significantly.

2. The amino acid composition of the *N*-terminal peptides has been determined. The oxidized hormone differs in the substitution of methionine sulphoxide for the methionine present in the untreated material.

3. The amino acid composition of Raney-nickel-treated corticotrophin has been investigated after leucine aminopeptidase hydrolysis. Contrary to the findings of earlier studies on acid hydrolysates, methionine is still present in the material.

4. On the basis of these studies and the correlation between biological activity and the extent of oxidation of methionine, it is concluded that the oxidation–reduction centre of corticotrophin A_1 is the thioether grouping of methionine.

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Comparative Studies of 'Bile Salts'

13. BILE ACIDS OF THE LEOPARD SEAL, *HYDRURGA LEPTONYX*, AND OF TWO SNAKES OF THE GENUS *BITIS**

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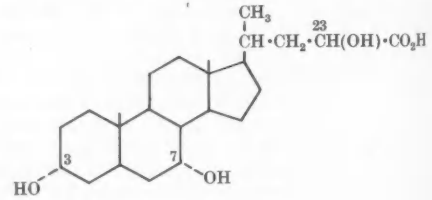
In the preliminary survey with which this work began the bile of ten species of snakes was examined. The Boidae (three species) gave pythocholic lactone (3α:12α:16α-trihydroxycholanic acid lactone), six other species yielded cholic acid (3α:7α:12α-trihydroxycholanic acid), but from the Gaboon viper, *Bitis gabonica*, no known bile acid was isolated (Haslewood & Wootton, 1950). An investigation of the bile salts of this species by paper chromatography (Haslewood & Sjövall, 1954) confirmed their unusual nature.

The work now reported included a detailed chemical examination of the bile salts of the Gaboon viper and also those of the puff adder, *Bitis arietans*, a closely related species. It was found that the bile salts of these snakes resembled those of Pinnepedia. For comparison, therefore, the leopard seal, *Hydrurga leptonyx*, was also investigated. Some knowledge was, incidentally, obtained of the bile acids of the Californian sealion, *Zalophus californianus*.

RESULTS

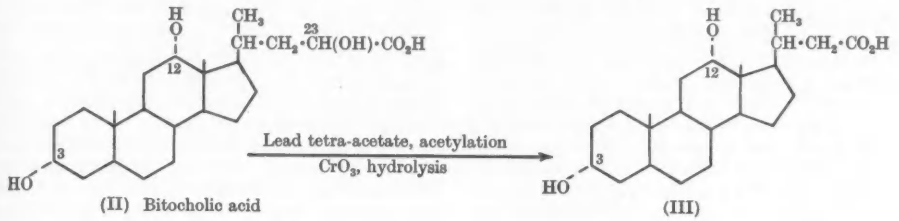
Paper chromatograms of the methyl or ethyl esters of Gaboon-viper bile acids showed faint spots corresponding to those given by methyl or ethyl cholate, together with two additional intense spots. In the solvent systems used, one of these intense spots (P) appeared just behind the methyl or ethyl cholate spot, and the other (Q) moved only a little way from the start-line. Fortuitously it was noticed that spots P and Q appeared at the same places on chromatograms as spots given by the corresponding esters of leopard-seal and Californian-sealion bile acids. Chromatography on alumina of the ethyl esters of Gaboon-viper bile acids easily separated the fraction responsible for spot P from a 'spot Q–ethyl cholate' fraction, described later.

The ethyl ester giving spot P was hydrolysed and the (non-crystalline) acid was investigated, with the clue that it might be related to the bile acids of Pinnepedia. The characteristic chemical feature of these acids is the C-23 hydroxyl group, found by Windaus & van Schoor (1928) in 'β-phocaecholic acid' (3α:7α:23-trihydroxycholanic acid, I). This acid was now isolated from leopard-seal and Californian-sealion bile and conditions were found by which the $-CH(OH)\cdot CO_2H$ grouping could be quantitatively estimated by lead tetra-acetate oxidation. Application of this method to the 'spot P' of Gaboon-viper acid gave the same result, quantitatively, as had been obtained from 'β-phocaecholic acid' (I). However, the 'spot P' acid did not crystallize with (I), and its specific rotation was $[\alpha]_D + 48{\cdot}5°$, in contrast with $[\alpha]_D + 11°$ for 'β-phocaecholic acid'. These facts, and the



(I)

* Part 12: Haslewood & Ogan 959).



(II) Bitocholic acid (III)

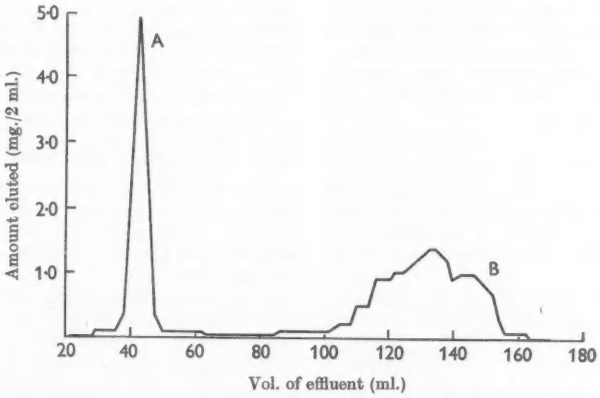


Fig. 1. Results of separating the complex (40 mg., m.p. about 205°) of ethyl esters from Gaboon-viper bile by partition chromatography on Celite (10 g.). Peak A is due to the ethyl ester of an isomer of cholic acid and peak B to ethyl 3α:7α:12α:23-tetrahydroxycholanate. Details are as given in the text.

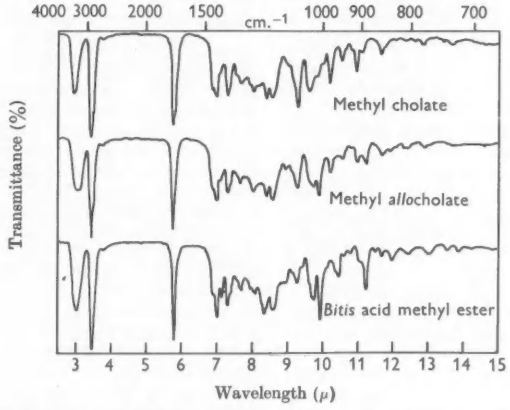


Fig. 2. Infrared spectra in KBr of methyl cholate, methyl *allo*(5α)cholate and the methyl ester of the cholic acid isomer, from Gaboon-viper and puff-adder bile, whose ethyl ester gave peak A (Fig. 1). Spectra were recorded separately and are redrawn together for comparison. In each case the percentage transmittance at 2·5 μ was above 94.

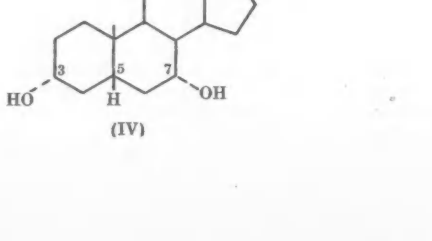
finding that the methyl or ethyl esters of the 'spot P' acid ran on chromatograms at the same rate as the corresponding esters of (I), suggested that the new acid might be 3α:12α:23-trihydroxycholanic acid (II), which would be expected to have the properties described. A larger sample of the acid giving spot P was therefore oxidized with lead tetra-acetate and the product was acetylated and further oxidized with chromic acid. From the saponified product there was obtained nordeoxycholic acid (III), identical with a sample made from deoxycholic acid (3α:12α-dihydroxycholanic acid) by the method of Riegel, Moffett & McIntosh (1944). These experiments show that a chief constituent of Gaboon-viper bile acids is (II), which it is proposed to call *bitocholic acid*. Methyl bitocholate was readily obtained crystalline as its complex with diethyl ether.

For confirmation of the formula (I) of Windaus & van Schoor (1928), 'β-phocaecholic acid' was degraded by the above-described methods (as II → III) to give norchenodeoxycholic acid, also prepared by Wieland–Barbier degradation of chenodeoxycholic acid (3α:7α-dihydroxycholanic acid).

The 'spot Q–ethyl cholate' fraction mentioned above was crystallized from ethyl acetate to give a substance melting constantly at about 205° and giving an elementary analysis corresponding to a formula $C_{26}H_{44}O_5$ or $C_{26}H_{44}O_6$. Paper chromatography of this substance showed it to be a complex containing an ester with the mobility of ethyl cholate and another giving spot Q. Separation on Celite gave the result shown in Fig. 1.

Elution peak A (Fig. 1) was due to an isomer (m.p. 225°, $[\alpha]_D + 23°$) of ethyl cholate which gave a positive Hammarsten (HCl) test. The corresponding methyl ester had m.p. about 227° and the acid melted at about 240°. The infrared spectra of the new methyl ester, of methyl *allo*(5α)cholate and of methyl cholate are shown in Fig. 2.

Peak B (Fig. 1) was given by an ester which crystallized as a hydrate, m.p. 182°, $[\alpha]_D + 36°$, and on saponification gave an acid with the properties expected of 3α:7α:12α:23-tetrahydroxycholanic acid (IV). Bergström, Krabisch & Lindeberg (1959) claim that the 'α-phocaecholic acid' of Hammarsten (1909, 1910) has this constitution. The infrared spectrum of the 'α-acid' was almost but not quite identical with that of the tetrahydroxy acid from the Gaboon viper. Probably both the acid of Bergström *et al.* (1959) and the Gaboon viper tetrahydroxy acid consist mainly of 3α:7α:12α:23-tetrahydroxycholanic acid (IV), and elution peak B (Fig. 1) is due to ethyl 3α:7α:12α:23-tetrahydroxycholanate. Some evidence was obtained that Gaboon-viper bile contained a little cholic acid.



(IV)

An identical complex, m.p. about 205°, was isolated from the ethyl esters of puff-adder bile acids and was resolved in the same way and with the same results as shown in Fig. 1. The bile of the puff adder also yielded methyl bitocholate.

Leopard-seal and Californian-sealion bile yielded 'β-phocaecholic acid', and from the leopard-seal ethyl esters there was obtained a complex, m.p. about 205°, having almost the same elementary analysis and infrared spectrum as the corresponding substance from the Gaboon viper. Resolution of this complex on Celite gave a result very similar to that shown in Fig. 1, and the ester corresponding to elution peak B (Fig. 1) was also ethyl 3α:7α:12α:23-tetrahydroxycholanate. The ester eluted at the position of peak A (Fig. 1) was, however, ethyl *allo*(5α)cholate [ethyl 3α:7α:12α-trihydroxy(5α)-cholanate], previously prepared from cholic acid (Anderson & Haslewood, 1960), and there was no evidence for the presence of the ester, m.p. 225°, described above.

Attempts to detect the value and sign of the contribution to $[\alpha]_D$ of the ·CH(OH)· group at C-23 in 'β-phocaecholic acid' by comparing the specific rotations of this acid, its ethyl ester and its acetylated ethyl ester with the rotations of the corresponding derivatives of chenodeoxycholic acid failed, since, within the limits of experimental error, the contribution to $[\alpha]_D$ of the asymmetry at C-23 could not be detected.

EXPERIMENTAL

General. Details were as described by Haslewood & Wootton (1951), except that melting-point determinations and esterification of bile acids were done as by Haslewood (1956). Paper chromatography was as described by Haslewood (1956), and partition chromatography on Celite as by Haslewood & Ogan (1959). Infrared spectroscopy in the range 2·5–15·0 μ was done with a Perkin-Elmer Infracord apparatus, by the KBr-disk method. A solution of the steroid (0·5–1·0 mg.) in acetone (about 0·2 ml.) was dropped on to powdered KBr (320 mg., Merck's 'for infrared spectroscopy') in a mortar and the solvent was evaporated at 50°. The KBr was re-ground and a disk prepared in the usual way. The use of acetone ensured the removal of the solvent frequently present in crystalline bile acids and their derivatives.

Lead tetra-acetate oxidation (quantitative method). The substance to be tested (1·0–4·0 mg.) was dissolved at about 50° in 1 ml. of 0·01 M-lead tetra-acetate in acetic acid, a blank (without substance) being prepared at the same time. After 16–18 hr. at 54°, there was added to each mixture 1 ml. of an aqueous solution containing sodium acetate trihydrate (50 g.) and KI (2 g.)/100 ml. (Baer, 1940). The liberated iodine was titrated with 0·01 N-$Na_2S_2O_3$. With this method, phocaecholic acid ('β-phocaecholic acid') consumed, compared with the blank, 1 equiv. of oxygen (error in three successive determinations, 0–+0·3%).

Preparation of esters. Bile salts (1 g.) were hydrolysed by heating in a small bomb at 114±2° for 8–16 hr. with 2·5 N-KOH (10 ml.). The contents of the bomb were washed out with water and treated with 2 N-HCl and NaCl (excess). After some hours at 0–5°, the solid crude bile acids were collected, washed with water and esterified as described above.

Separation of the ethyl esters of Gaboon-viper and puff-adder bile acids

The description below applies to both species, except where otherwise stated.

Preliminary paper chromatography. The ethyl esters in system B_3 of Bush (1952) gave a faint spot at the same place as ethyl cholate, a strong spot (P), just behind this and another (Q) just moving from the start-line. Neither P nor Q appeared at the same place as spots given by pythocholic lactone or ethyl varanate (Haslewood & Wootton, 1950) run on the same chromatogram. Similar results were given by the methyl esters of Gaboon-viper bile acids, except that spot P appeared at the same place as the spot due to pythocholic lactone. P and Q, whether due to ethyl or methyl esters, appeared at the same places as spots derived from the corresponding esters of leopard-seal or Californian-sealion bile acids.

Separation of ethyl esters on alumina. The esters (0·5 g.) in benzene (about 30 ml.) were put on to a column of Al_2O_3 (5 g.) made up in benzene. Elution was as follows (eluting solvent, ml., mg. eluted): benzene, 1000, 215; ether, 250, 12; ethanol, 250, 234. Total eluted, 461 mg. Paper chromatography showed that the benzene-eluted fraction gave only spot P, and that this was missing from the ethanol ('ethyl cholate–spot Q') fraction. In subsequent work, only the 'spot P' and 'ethyl cholate–spot Q' fractions were used, the small fractions eluted by ether being discarded.

Bitocholic acid. A fraction (194 mg.) giving only spot P, eluted by benzene as described above, was boiled under reflux for 40 min. in ethanol (3 ml.) with 5 N-KOH (0·5 ml.). Ethanol was removed by evaporation in N_2 and a solution of the residue in water was filtered. The filtrate was treated with 2 N-HCl and NaCl (excess). After cooling to 0–5° overnight, the solid acid was collected, washed with water and dried by evaporation with ethanol *in vacuo*. Yield, 171 mg. This bitocholic acid formed a colourless 'solid foam' which gave the same result as phocaecholic acid in the lead tetra-acetate method, but would not crystallize on seeding in ethyl acetate with this acid. Treatment of crude bitocholic acid in methanol with diazomethane (excess), followed by evaporation of solvent, left a gum which readily crystallized from ether as large colourless prisms of the ether complex of *methyl bitocholate*, m.p. 114–123°; $[\alpha]_D^{23}$ +44·2±1° in ethanol (*c*, 0·34) (Found: C, 70·4; H, 10·2. $C_{25}H_{42}O_5,C_4H_{10}O$ requires C, 70·2; H, 10·5%). This substance would crystallize only as the complex from ether: it gave a single spot as 'spot P' on paper chromatograms. Saponification of crystalline methyl bitocholate complex gave bitocholic acid, finally as a colourless gum with $[\alpha]_D^{23}$ +48·5±1° in ethanol (*c*, 1·3), which gave no colour in the Hammarsten HCl test.

Isolation and resolution of the complex m.p. about 205°. Combined fractions (497 mg.) of ethyl esters eluted by ethanol from Al_2O_3 in the separation described above were crystallized twice from ethyl acetate to give fluffy white needles of m.p. 202–206° (Found: C, 70·0; H, 9·7. $C_{26}H_{44}O_5$ requires C, 71·6; H, 10·1; $C_{26}H_{44}O_6$ requires C, 69·0; H, 9·7%). On paper chromatography with the solvent system G_3 of Haslewood & Sjövall (1954) this substance gave a spot at the same place as ethyl cholate (R_F 0·79) and another (Q) with R_F 0·24. The mixture (40 mg.) was separated on Celite with the system (EC_1) ethanol–water–light petroleum (b.p. 80–100°)–benzene (10:4:7:7, by vol.). Celite (Johns-Manville Co. Ltd., London) (10 g.) was impregnated with stationary (ethanol–water) phase (5 ml.), and, after packing, the above-mentioned complex in stationary phase (0·5 ml.) was put on to the column as described by Haslewood & Ogan (1959). The eluate with the moving phase was automatically collected in fractions (2 ml. each) into weighed tubes, with the result shown in Fig. 1.

Isolation of substance giving peak A (Fig. 1). Fractions eluted by between 40 and 46 ml. (both inclusive) were combined and the total crystalline residue (13 mg.) was recrystallized from aqueous ethanol–acetone to give white leaflets (10·5 mg.) of m.p 224–226°; $[\alpha]_D^{21}$ +23±2° in ethanol (*c*, 1·1) (Found: C, 71·75; H, 10·3. $C_{26}H_{44}O_5$ requires C, 71·6; H, 10·1%). This *substance*, like ethyl cholate, gave a purple colour in the Hammarsten HCl test. It (3 mg.) on saponification gave the corresponding acid, which slowly formed, from ethyl acetate, long needles of m.p. 239–241° (decomp.). The methyl ester, made with diazomethane, crystallized from ether as needles of m.p 224–227°: its infrared spectrum is shown in Fig. 2. It ran on paper chromatograms, in system G_3, at the same rate as methyl cholate and methyl *allo*cholate. There was insufficient pure material for further elementary analyses.

Isolation of substance giving peak B (Fig. 1). Fractions eluted by between 110 and 152 ml. (inclusive) were combined. The product (27 mg.) from (undried) ethyl acetate formed a gel which slowly became fine colourless needles of m.p. 184–185°; $[\alpha]_D^{27}$ +36±1° in ethanol (*c*, 1·1) (Found: C, 67·3; H, 9·4. $C_{26}H_{44}O_6,\frac{1}{2}H_2O$ requires C, 67·7; H, 9·8%). This (probably) *ethyl* 3α:7α:12α:23-*tetrahydroxycholanate* gave a greenish purple in the Hammarsten–HCl test. Saponification gave an amorphous acid, which consumed 1 equiv. of oxygen in the lead tetra-acetate method and gave an infrared spectrum almost identical with that of a specimen of Hammarsten's 'α-phocaecholic' acid supplied by Professor S. Bergström.

Evidence for cholic acid in Gaboon-viper bile. The ethyl acetate liquors from which the complex, m.p. about 205°, had been crystallized were evaporated, leaving a gelatinous solid. When this (50 mg.) was separated on Celite as described above there was obtained, in the place corresponding to peak A (Fig. 1), a substance (3·5 mg.) which had m.p. about 160° and appeared to be an impure sample of ethyl cholate. The substance (44·4 mg.) recovered in the peak B region (Fig. 1) was apparently ethyl 3α:7α:12α:23-tetrahydroxycholanate.

It could be calculated that constituents of the Gaboon-viper bile acid esters were present approximately in the following proportions (by wt.): ethyl bitocholate, 43%; ethyl 3α:7α:12α:23-tetrahydroxycholanate, 44%; ester m.p. 225°, 3%; ethyl cholate, 3%.

Bile acids of the leopard seal and Californian sealion

Leopard-seal bile acids and esters. The bile salts (8·2 g. from one gall-bladder) consisted of a non-deliquescent buff solid. When this (1 g.) was hydrolysed as described above, the crude bile acids (0·75 g.) formed an almost white solid. Esterification of this (3·77 g.) as previously described gave ethyl esters (3·16 g.), which, after treatment with Girard T reagent (Haslewood, 1956), yielded 'ketones' (0·02 g.) and 'non-ketones' (2·92 g.). The gummy 'ketones' were not further investigated.

Separation of ethyl esters on alumina. The esters (1·89 g.) in benzene (about 20 ml.) were put on to Al_2O_3 (19 g.) in a column. Benzene (1 l.) eluted 1·10 g., ether (250 ml.) eluted 0·05 g. and ethanol (250 ml.) eluted 0·64 g. Total eluted, 1·79 g. Paper chromatography, in Bush's (1952) system *A*, showed that the fractions eluted by benzene and ether both gave a strong spot (P) corresponding to ethyl phocaecholate and a faint spot running slightly more slowly than ethyl chenodeoxycholate. In Bush's (1952) system B_3, the fraction eluted by ethanol gave a strong spot (Q) running much more slowly than ethyl cholate and a faint spot at the same place as ethyl cholate.

Isolation and properties of phocaecholic acid. Combined benzene and ether eluates (1·70 g.) were heated for 20 min. under reflux in ethanol (50 ml.) with 5N-KOH (3 ml.). Ethanol was evaporated *in vacuo* and the residue was dissolved in water and treated with 2N-HCl and NaCl (excess). After cooling to 0–5°, the solid acids were collected, washed and crystallized twice from ethyl acetate, to give phocaecholic acid (0·5 g.), final m.p. 222–224°; $[\alpha]_D^{24} + 10{\cdot}8 \pm 1°$ in ethanol (*c*, 2·8). Ethyl phocaecholate (not crystalline) had $[\alpha]_D^{24} + 10{\cdot}4 \pm 1°$ in ethanol (*c*, 2·7). This ester (32 mg.) was left in pyridine (0·5 ml.) with acetic anhydride (0·5 ml.) for 2 days. The mixture was diluted with 2N-HCl and the product was extracted twice with ether. The extract was washed with water, aqueous ammonia and water and dried (Na_2SO_4). Evaporation left a crystalline residue of *ethyl triacetyl phocaecholate*, which gave large crystals, m.p. 165–171°, from light petroleum, and from aqueous ethanol gave small white hydrated needles, becoming amorphous at 144°, then crystallizing and finally melting at about 170°; $[\alpha]_D^{22} + 18{\cdot}7 \pm 1°$ in ethanol (*c*, 2·0) (Found, after drying: C, 68·2; H, 9·2. $C_{32}H_{50}O_8$ requires C, 68·3; H, 8·9%). Crystalline methyl chenodeoxycholate had $[\alpha]_D^{20} + 11{\cdot}2 \pm 1°$ in ethanol (*c*, 2·8) and the diacetate of this ester had $[\alpha]_D^{22} + 17{\cdot}5 \pm 1°$ in ethanol (*c*, 2·1). Corresponding fractions from Al_2O_3 prepared in this laboratory by Dr R. J. Bridgwater of the ethyl esters of Californian-sealion bile acids similarly yielded phocaecholic acid.

Complex m.p. about 205° from the leopard seal. The fraction of ethyl esters eluted from Al_2O_3 by ethanol (above) was crystallized twice from ethyl acetate, from which it gave long white needles, m.p. 201–204°, not depressed by the corresponding substance from Gaboon-viper bile (Found: C, 69·6; H, 10·1%). The compound had almost the same infrared spectrum as the Gaboon-viper substance and 13 mg. was separated on Celite exactly as described above. The peak A (Fig. 1) ester (5 mg.) appeared from aqueous ethanol as a gel, turning gradually into fine white needles, m.p. 204–205°; Hammarsten test, blue. Its infrared spectrum was identical with that of ethyl *allo*cholate, similarly separated from a complex with ethyl 3α:7α:12α:23-tetrahydroxycholanate (Anderson & Haslewood, 1960). The peak B (Fig. 1) ester (8 mg.) from the leopard-seal complex formed from ethyl acetate a gel which became long white needles of m.p. 181–183°, not depressed by ethyl 3α:7α:12α:23-tetrahydroxycholanate isolated as described above from snake bile.

Conversion of bitocholic acid into nordeoxycholic acid

Bitocholic acid (38 mg.) was dissolved in 3 ml. of 0·05M-lead tetra-acetate in acetic acid. The solution was kept at 50° for 18 hr., after which time iodine was liberated with the sodium acetate–KI reagent (3 ml.) described above. Titration showed that more than 1 equiv. of oxygen had been consumed. Excess of 0·05N-$Na_2S_2O_3$ and NaCl were added. The product was extracted twice with ether and the extract washed with water and dried (Na_2SO_4). Evaporation left an almost colourless gum (40 mg.), which was dissolved in acetic acid (1 ml.) with acetic anhydride (0·2 ml.). The solution, cooled to about 20°, was treated with 8·5N-perchloric acid (1 drop). After 10 min., the mixture was diluted with water and the product extracted twice with ether. The ether was washed with water, dried (Na_2SO_4) and evaporated. The residue (45 mg.) in acetic acid (1 ml.) was oxidized for 15 min. at about 20° with 20% CrO_3 (0·1 ml.). Water and NaCl (excess) were added and the product was extracted twice with ether. Evaporation of the washed and dried extract left a gum, which was boiled under reflux for 45 min. with water (5 ml.) and 2N-KOH (1 ml.). The cooled mixture was filtered and the filtrate treated with 2N-HCl and NaCl (excess). The amorphous solid was collected, washed and dried by evaporation *in vacuo* with ethanol. The product (23 mg.) was separated on Celite (10 g.) with the system (EC_2) light petroleum (b.p. 80–100°)–water–ethanol (10:1:9, by vol.). After equilibration, the lower (stationary) phase (5 ml.) was put on to the Celite. The separation was carried out exactly as described above for the complex, m.p. 205°. Moving phase (28–58 ml.) eluted a broad band of material (12·2 mg.) giving on paper chromatograms a single spot corresponding to ethyl nordeoxycholate. This material was hydrolysed by warming for 20 min. with ethanol (0·2 ml.) containing 5N-KOH (0·05 ml.). Ethanol was evaporated in N_2 and the residue dissolved in water. The filtered solution was acidified with 2N-HCl and the solid acid was collected, washed and crystallized from acetone, from which it gave large colourless crystals (6 mg.) of m.p. about 140 and 209–211°, not depressed by authentic nordeoxycholic acid (m.p. about 150 and 210–212°) prepared by the method of Riegel *et al.* (1944). A sample of each acid was methylated with diazomethane and the infrared spectra of the products were found to be identical.

Conversion of phocaecholic acid into norchenodeoxycholic acid

Phocaecholic acid (40 mg.) was dissolved in 4 ml. of 0·05M-lead tetra-acetate in acetic acid. After 17·5 hr. at 49°, the mixture was diluted with sodium acetate–KI

reagent (4 ml.) and iodine was titrated with 0·05 N-$Na_2S_2O_3$; NaCl (excess) was added and the product extracted twice with ether. The ether was washed with water, dried (Na_2SO_4) and evaporated. The residue was left for 65 hr. in pyridine (1 ml.) with acetic anhydride (1 ml.). The solution was diluted with 2 N-HCl and the product recovered with ether as before. Evaporation of the washed and dried ether left a gum which was dissolved in acetic acid (0·5 ml.) and oxidized with 20% CrO_3 (0·2 ml.) at about 20° for 20 min. After treatment with water and NaCl (excess) the product was recovered with ether as before. Evaporation of the washed and dried ether left a residue which was gently warmed and shaken with dilute aqueous $NaHCO_3$. The solution was filtered and the small insoluble residue and filter were washed with water. The combined filtrate and washings were acidified with HCl and the precipitated acid was collected, washed, dissolved in acetone and evaporated to dryness *in vacuo*. The residue was converted into ethyl esters as described above. The partly crystalline esters (26 mg.) were purified by elution from Al_2O_3 (0·4 g.) in a column with benzene (100 ml.). The residue (21 mg.) obtained by evaporating the benzene had an indefinite m.p. and was re-acetylated by dissolution in acetic acid (0·5 ml.) with acetic anhydride (0·1 ml.), followed by treatment with 8·5 N-perchloric acid (1 drop). After 20 min. the mixture was diluted and the product was recovered with ether as before. Evaporation of the washed ether left a crystalline residue (20 mg.), which from light petroleum gave colourless prisms, m.p. 128–133°, and separated from aqueous ethanol as long prisms of m.p. 131–133°, not depressed by authentic ethyl diacetyl norchenodeoxycholate and showing the same infrared spectrum as this substance.

Preparation of norchenodeoxycholic acid

To a Grignard reagent made from bromobenzene (1·2 ml.), magnesium (0·25 g.) and ether (25 ml.) was added, with shaking, a solution of methyl chenodeoxycholate (0·25 g.) in benzene (8 ml.). The mixture was heated under reflux for 1 hr. and then poured on to a mixture of 10 N-HCl and ice. The product was extracted twice with ether and the extract was washed with water, 2 N-KOH and water and was dried (Na_2SO_4). Evaporation left a residue which was suspended in water, through which steam was passed for 40 min. After cooling, the material not volatile in steam was collected and dried by evaporation *in vacuo* with ethanol–benzene. The residue was boiled under reflux for 1 hr. with a mixture of acetic acid (2·5 ml.) and acetic anhydride (1·25 ml.). Part (2 ml.) of this mixture was then distilled off and the remainder was diluted with water and extracted three times with ether. The extract was washed with water, aqueous ammonia and water and dried (Na_2SO_4). Evaporation left a brown gum (0·39 g.) which was dissolved in acetic acid (2 ml.) and oxidized at 50±3° with a solution of CrO_3 (0·3 g.) in 3·3 ml. of water. After 20 min. at 50±3°, the solution was poured into a saturated solution of NaCl and extracted three times with ether. The ether was washed with water, N-KOH and water. The combined alkaline washings were heated at about 80° in a stream of N_2 to expel ether, cooled and treated with 2 N-HCl and NaCl (excess). After cooling to about 0°, the white solid precipitate was collected and dried by evaporation *in vacuo* with acetone. The residue (0·11 g.) was esterified with ethanol as described above; the product (98 mg.) gave hydrated needles of m.p. 67–74° from light petroleum–benzene. These (50 mg.) were acetylated for 15 min. in acetic acid (1 ml.) with acetic anhydride (0·2 ml.) and 8·5 N-perchloric acid (1 drop). After addition of water (excess) a crystalline solid separated, which was collected, washed and crystallized twice from aqueous ethanol, from which *ethyl diacetyl norchenodeoxycholate* gave long colourless prisms of m.p. 131–134°; $[\alpha]_D^{25}$ +18·7±1° in ethanol (*c*, 1·6) (Found: C, 71·6; H, 9·3; $C_{29}H_{46}O_6$ requires C, 71·0; H, 9·4%). This ester (27 mg.) in ethanol (1 ml.) was warmed for 35 min. under reflux with 5 N-KOH (0·1 ml.). Ethanol was removed in N_2 and the residue dissolved in water and treated with 2 N-HCl and NaCl (excess). The precipitated acid was collected, washed and crystallized from aqueous ethanol, from which it formed long white needles, which appeared to lose solvent before melting, with decomposition, at 199–202°. This *norchenodeoxycholic acid* had $[\alpha]_D^{23}$ +12·6±1° in ethanol (*c*, 1·6) (Found: C, 71·4; H, 10·0; $C_{23}H_{38}O_4,\frac{1}{2}H_2O$ requires C, 71·3; H, 10·1%).

DISCUSSION

Nomenclature. Hammersten (1909, 1910) described as 'β-phocaecholic acid' a substance shown by Windaus & van Schoor (1928) to be probably (I). This constitution is confirmed by the present work. Hammarsten also found an 'α-phocaecholic acid' of unknown structure. Bergström *et al.* (1959) state that some of Hammarsten's original preparations consist almost entirely of 3α:7α:12α:23-tetrahydroxycholanic acid (IV). The acid (IV) cannot, however, be called 'α-phocaecholic acid', for it is not a cholic (trihydroxycholanic) acid in the sense used until the present time in bile acid nomenclature. It is proposed that Hammarsten's 'β-phocaecholic acid' be called 'phocaecholic acid'. The acid isomeric at C-23 will then be 23-*iso*phocaecholic acid, and can, like Hammarsten's 'β-acid', be eventually named in full as a trihydroxycholanic acid. The 3α:7α:12α:23-tetrahydroxycholanic acid of Bergström *et al.* and of the present work can be so named, or can be called 12α-hydroxyphocaecholic acid if it is shown to have the same configuration at C-23 as phocaecholic acid itself.

Chemical. (i) Cholic acid isomer whose ethyl ester gave peak A (Fig. 1). This substance is not *allo*(5α)cholic acid, but the infrared spectrum (Fig. 2) of its methyl ester shows two bands at about 9·73 and 9·91 μ, very similar in relative intensity to those at about the same wavelengths as are given by methyl *allo*cholate. A constitution which would possibly account for the other properties (specific rotation, behaviour on chromatograms, response to the Hammarsten test) of the new acid is that of 3β:7α:12α-trihydroxy*allo*(5α)-cholanic acid. Alternatively, the new substance may be a trihydroxy derivative of a hitherto undescribed carbon-skeleton isomer of cholanic acid.

(ii) Tetrahydroxy acid whose ethyl ester gave peak B (Fig. 1). This acid is undoubtedly substantially the same as the 'α-phocaecholic acid' of Bergström *et al.* (1959). Their evidence for its constitution as (IV) was based to an important extent on its conversion into norcholic acid. However, the optical rotation given ($[\alpha]_D + 22{\cdot}9°$ in ethanol) does not agree with the proposed structure, and the published melting point (152°) of the derived norcholic acid is not that (about 183°) obtained in this laboratory or published in the literature (Shimizu & Kazuno, 1936; Morsman, Steiger & Reichstein, 1937) for this substance.

The optical rotation ($[\alpha]_D + 20{\cdot}5°$ in ethanol) given by Bergström *et al.* (1959) for 'β-phocaecholic acid' also does not agree with the expected value (given in the present report) and perhaps the rotation figures in the paper of Bergström *et al.* should be discounted. Although the norcholic acid isolated may have contained impurities, as its melting point suggests, the fact that its mass spectrum was identical with that of authentic norcholic acid suggests that it most probably consisted essentially of this compound. From other experience, the writer has reason to believe that contamination with *allo*(5α)norcholic acid would not necessarily be detected in the mass spectrum. Such contamination would probably not be removed by partition chromatography and might have resulted from the presence of some *allo*(5α) acid in the original tetrahydroxycholanic acid. The writer's specimen of this acid might also be similarly contaminated, and such impurities would be sufficient to account for the small differences in infrared spectra between the Swedish material and that described here. Another source of differences might be the presence of different proportions of optical enantiomers at C-23. In summary, the evidence is strongly in favour of the view that the 'α-acid' of Bergström *et al.* (1959) is, in fact, 3α:7α:12α:23-tetrahydroxycholanic acid (IV), and that this is also the constitution of the corresponding acids from leopard seal and the snake bile now examined. In none of the acids hydroxylated at C-23 described has the optical configuration been elucidated, and it cannot even be confidently stated that they are, in fact, specimens of single optical enantiomers at this centre.

Biological. The two snakes of the genus *Bitis* comprise the only animals, except Pinnipedia, so far examined which have been shown to have bile acids hydroxylated at C-23. This chemical feature seems, by its distribution, to be a true species character and not an artifact of intestinal micro-organisms. If this is so, the livers of the species concerned presumably contain a C-23-hydroxylating-enzyme system, the presence of which should be detectable by direct experiment.

Apart from C-23 hydroxylation, the bile salts of the *Bitis* snakes and of the leopard seal are not more closely similar than those of other unrelated animals. In this seal, the chief bile acid is phocaecholic acid, which may, like chenodeoxycholic acid, be a 'primary' bile acid, made by the liver from cholesterol: 3α:7α:12α:23-tetrahydroxycholanic acid is a minor constituent. On the other hand, bitocholic acid, lacking a hydroxyl group at C-7, is, like deoxycholic acid, likely to be an artifact, made during the enterohepatic circulation by intestinal micro-organisms from 3α:7α:12α:23-tetrahydroxycholanic acid, which in these snakes is present in major amounts.

The *allo*cholic acid found in leopard-seal bile is possibly derived from fishes or penguins eaten, for these are likely to have contained this substance (Anderson, Haslewood & Wootton, 1957; Anderson & Haslewood, 1960). A discussion of the possible biological implications of the presence of the *iso*-cholic acid now newly found in snake bile must await the elucidation of its chemical constitution. It seems unlikely to be a dietary artifact, for Dr Osman Hill, who supplied the bile, states that the snakes had been fed on newborn rats and mice and chicks, the bile of which is not known to contain such an acid.

If the peculiar chemical nature of the bile salts of the Gaboon viper and puff adder is shared only by the few other snakes of the genus *Bitis*, consideration might be given to the more definite separation of this group of reptiles from the remainder of the Viperidae.

SUMMARY

1. Bile salts of the leopard seal, *Hydrurga leptonyx*, and of the Californian sealion, *Zalophus californianus*, have been shown to contain the 'β-phocaecholic acid' of Hammarsten (1909, 1910), which it is now proposed to call 'phocaecholic acid'. Leopard-seal bile was shown to contain also *allo*(5α)cholic acid and the 3α:7α:12α:23-tetrahydroxycholanic acid identified by Bergström *et al.* (1959) as Hammarsten's (1909, 1910) 'α-phocaecholic acid'. It is suggested that this name is inapplicable, as the substance is not a 'cholic' (trihydroxycholanic) acid.

2. Bile salts of the Gaboon viper, *Bitis gabonica*, and puff adder, *Bitis arietans*, contained a new acid, called 'bitocholic acid', an unidentified isomer of cholic acid and the same 3α:7α:12α:23-tetrahydroxycholanic acid as was found in seal bile.

3. Bitocholic acid was shown by its properties and by degradation to the known nordeoxycholic acid (3α:12α-dihydroxynorcholanic acid) to be 3α:12α:23-trihydroxycholanic acid.

4. Phocaecholic acid was degraded to norchenodeoxycholic acid (3α:7α-dihydroxynorcholanic acid), also prepared by Wieland–Barbier degradation from chenodeoxycholic acid. This work confirms the constitution suggested for phocaecholic acid by Windaus & van Schoor (1928).

5. In none of the C-23-hydroxylated bile acids described could the optical configuration at C-23 be determined by measurements of $[\alpha]_D$.

6. Although snakes of the genus *Bitis* contain C-23-hydroxylated bile acids, previously thought to be confined to the Pinnipedia, the types of bile acids present do not indicate a very close similarity between the bile salts of these snakes and those of Pinnipedia.

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The Breakdown of Chlorophyll by Chlorophyllase

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The first stage in the enzymic breakdown of chlorophyll *in vivo* may be the removal of the phytol side chain; chlorophyllase, the enzyme which *in vitro* catalyses its removal and attachment, has been found in most plants. The enzyme was discovered by Willstätter & Stoll (1910), and Mayer (1930) made an extensive survey of chlorophyllase activity in different species and investigated some properties of the enzyme. Krossing (1940) found that it was in the chloroplast fraction of leaves. Chlorophyllase action *in vitro* usually requires the presence of a solvent such as acetone (40–70 %, w/v) or methanol (70–80 %), but Weast & Mackinney (1940) found that in a few species at some seasons of the year there was no hydrolysis in ethanol, though an enzyme system active in water at 70° was present. Peterson & McKinney (1938) investigated the effect of mosaic viruses on the chlorophyllase activity and chlorophyll content of tobacco leaves.

Dried leaves have frequently been used as a source of enzyme for preparing chlorophyllides from chlorophyll. Fischer & Lambrecht (1938) used a leaf-meal preparation for studying the specificity of chlorophyllase action with various chlorophyll derivatives.

Mayer (1930) found that little or no chlorophyllase activity was extracted from leaf powders by water, aqueous buffer solutions or glycerol. Willstätter & Stoll (1910) obtained a trace of soluble chlorophyllase by subjecting macerated fresh leaves to 250 atmospheres pressure. Ardao & Vennesland (1960) found that a soluble chlorophyll–lipoprotein complex obtained when spinach chloroplasts are treated with digitonin had chlorophyllase activity.

The present paper is concerned with the preparation of soluble chlorophyllase, its partial purification and some of its properties.

MATERIAL AND METHODS

Plant material

Sugar beet (*Beta vulgaris* var. *saccharifera*) leaves were obtained from experimental plots on Rothamsted Farm. The leaves could be kept at 0° in polythene bags for up to a

month with little loss of chlorophyllase activity. Seedlings of pea (*Pisum sativum* var. Sutton's Improved Pilot), bean (*Phaseolus vulgaris* var. Canadian Wonder), wheat (*Triticum vulgare* var. Capelle Desprez) and barley (*Hordeum vulgare* var. Proctor) were grown in a glasshouse either in moist sand in glass tanks or in compost in pots. Leaves of other species were from plants cultivated in local gardens or growing wild.

Preparation of enzyme-containing material

(1) Leaves were dried in an oven at 40° and ground in a Casella Grain Mill with 0·5 mm. plate. Although the powder contained both enzyme and substrate, chlorophyll was not converted into chlorophyllide until it was incubated in aqueous acetone. (2) Fresh leaves were ground in a Townson and Mercer Ltd. top-drive macerator in sufficient cold acetone to give a concentration of about 80%. The suspension was filtered on a Büchner funnel; the solid portion was washed with acetone to remove as much pigment as possible, dried at room temperature and then ground finely in a Casella mill.

Method of determining chlorophyllase activity

A number of methods have been proposed for determining chlorophyllase activity. One of several described by Willstätter & Stoll (1910), which was later used by Mayer (1930) and Peterson & McKinney (1938), is fairly satisfactory as a routine method. However, it requires extraction of pigment first into ether and then into alkali and an emulsion is often formed. A chromatographic method described by Sironval (1954) and used by Gage & Aronoff (1956) is suitable where great accuracy is required but is too slow and laborious for routine use. A method used by Weast & Mackinney (1940) based on the insolubility of chlorophyllides in light petroleum was comparatively simple and formed the basis for the method used in the present investigation and also for that described recently by Ardao & Vennesland (1960).

For determining the activity of fresh leaf and of dried leaf meal the substrate was the chlorophyll already present. For acetone-dried powders and soluble preparations the substrate was an acetone extract of fresh bean leaves which in addition to the chlorophylls contained xanthophylls and carotene. It was prepared by grinding the leaves in acetone (4 ml./g. of leaf), filtering rapidly through a Büchner funnel and then storing the extract at 0° for several days before use. Carotene which precipitated out during storage was removed by filtration. The solution had a chlorophyll content of about 0·5 g./l. as determined by the method of Arnon (1949).

Powders. A weighed sample of powder (up to 500 mg.) was incubated at room temperature in the dark with 8 ml. of chlorophyll solution and 4 ml. of sodium citrate solution, the final concentration of acetone being about 50% and of citrate 0·04 M; the suspension had a pH of about 8. Powders containing both enzyme and substrate were incubated under similar conditions but without added chlorophyll. After incubation for 1, 3 or 18 hr., depending on the activity of the preparation, the powder was filtered off on a Büchner funnel and washed with acetone until all pigment was extracted. To the filtrate 10 ml. of 2% (w/v) NaCl was added and the volume made up to 50 ml. with 80% acetone. Half the solution was kept for determination of the total chlorophyll present. The other half was shaken twice with 10 ml. lots of light petroleum (b.p. 40–60°) to extract the unchanged chlorophyll. The light petroleum was discarded and the acetone layer containing the chlorophyllides was again made up to 25 ml. with 80% acetone. The absorption of the two solutions was read in an EEL photoelectric colorimeter with filter 607. Even when the conversion of chlorophyll into chlorophyllide was complete the absorption maximum at 660 mμ was decreased by less than 5%, so there was no 'bleaching' due to enzymic action or other causes under the conditions used.

The enzyme activity was expressed as a percentage of the substrate split in a given time, 1 unit being defined as the amount which will catalyse the breakdown of 10% of the substrate to chlorophyllide in 1 hr.

Soluble preparations. The final concentration of acetone was 40% and the incubation period was 1 hr. or less. It was not necessary to filter before extraction with light petroleum.

Fresh leaf. A sample of fresh leaf was ground in ten times its weight of 50% acetone and the suspension was incubated for 1, 3 or 18 hr.

Nitrogen. This was determined by a micro-Kjeldahl method with SeO_2–$CuSO_4$–K_2SO_4 (1:1:8) catalyst.

EXPERIMENTAL AND RESULTS

Distribution and formation of chlorophyllase

Although Mayer's (1930) survey of activity in different species suggested possible useful sources for obtaining the enzyme in amounts sufficient for starting purification, many plants were examined in the course of investigation. The high activity of *Heracleum*, *Lamium* and *Dahlia* and the very low activity of *Urtica*, *Cucurbita* and *Phaseolus* was confirmed. Woody nightshade (*Solanum dulcamara*) had activity comparable with that of *Heracleum*. The leaves of both spinach and sugar beet (varieties of *Beta vulgaris*) had much higher activity than all other species tested. White bryony (*Bryonia dioica*), tomato (*Lycopersicum esculentum*), tobacco (*Nicotiana tabacum*) and Brussels sprout (*Brassica oleracea gemmifera*) all had low activity. Monocotyledons mostly appear to have lower activity than dicotyledons, though the activity of wheat was comparatively high. No activity was detected in the shoots of young seedlings of barley.

Chlorophyllase distribution in pea seedlings. Although the chlorophyllase activity of pea seedlings is low, they provide convenient material for studying changes in enzyme activity during germination and growth.

Dried peas were soaked in tap water overnight and samples (10 peas) were weighed and used for chlorophyllase determinations. The soaked peas were sown in moist sand and sampled at intervals for 14 days. The temperature of the glasshouse in which the peas were grown was 16–17° and the seedlings were in daylight for about 9 hr. daily. Artificial light was provided by 'cool white'

fluorescent tubes for a further 6 hr. daily. Duplicate lots were taken at each sampling and as soon as the embryos started to develop the seedlings were divided into cotyledons (with testas discarded), roots, stems and leaves, which were weighed separately. On some occasions samples were taken for dry-matter determination. For chlorophyllase estimation each portion was ground in acetone and the acetone-dried powder was incubated with chlorophyll for 18 hr. because of the low activity. Fig. 1 shows chlorophyllase changes during the first 2 weeks of growth. The enzyme activity in the soaked cotyledons was low; the activity doubled in the next 2 days and then fell again as the embryo developed. The radicle and roots were almost devoid of activity at every sampling. At 10 days 75% of the activity was in the shoot and most of it was in the leaves. The activity of the leaves expressed on a wet-weight basis rose sharply between the fifth and tenth days and then remained nearly constant although the amount per seedling was still increasing.

Effect of light on the chlorophyllase activity of pea seedlings. Seedlings grown in the dark had much lower activity than those in the light. At 10 days, leaves from light-grown seedlings had four times as much activity as dark-grown. When dark-grown seedlings were transferred to light the enzyme activity in the leaves increased (Fig. 2). The formation of enzyme paralleled the development of chlorophyll, and after 2 days both were only slightly less than in the light-grown seedlings.

In other experiments the seedlings were grown in compost instead of sand so that they could be kept longer. The activity of dark-grown seedlings after a small initial rise did not continue to increase after the fourteenth day, but it rose rapidly when seedlings were transferred to light even after 20 days in the dark.

Soluble enzyme preparations

A variety of methods (such as the use of detergents, incubation with other enzymes etc.) which have been used for making soluble preparations of other enzymes were tried in unsuccessful attempts to prepare soluble chlorophyllase from the leaves of several species. It was only when sugar-beet leaves had been found to provide a starting material of exceptionally high activity that a method of making a soluble enzyme preparation was found. The method does not, however, appear to be of general application; soluble preparations could not be obtained from *Heracleum* leaves nor from several other species which have moderately high activity. The activity in extracts from nightshade leaves was too low to be of any use.

Some activity could be extracted from an acetone-dried powder of sugar-beet leaves by a neutral or slightly alkaline buffer solution, but clarification of the extract by centrifuging or by filtration through diatomaceous earth removed

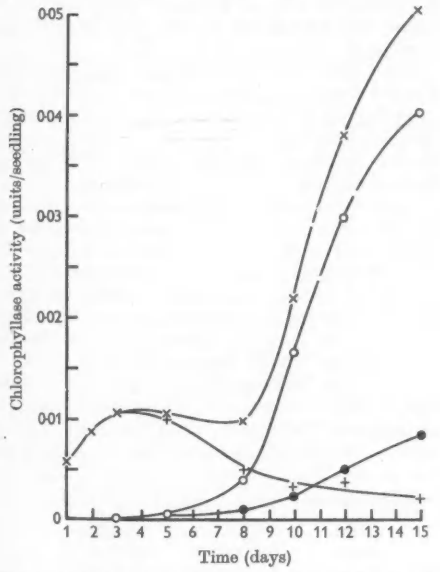

Fig. 1. Chlorophyllase activity during germination and growth of pea seedlings in the light. ×, Total; +, cotyledons; ○, leaves; ●, stem.

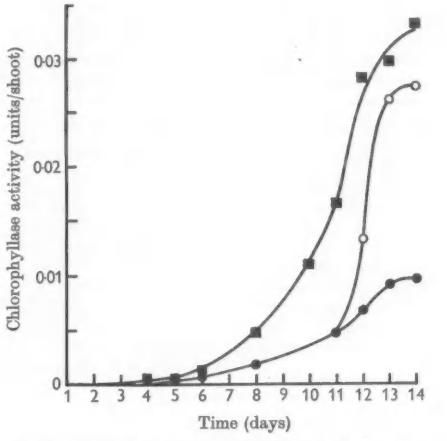

Fig. 2. Effect of light on chlorophyllase activity of pea-seedling shoots. ■, Grown in light; ●, grown in dark; ○, grown in dark and transferred to light after 11 days.

much of the activity. A soluble preparation could be made by extracting an acetone-dried powder first with 95% ethanol, followed by ethanol–ether (3:1) and finally ether, and then soaking the powder in 0·02 M-sodium citrate solution (8 ml./g. of powder) at room temperature overnight. The extract was squeezed out through cotton cloth and the residue re-extracted with citrate solution (2 ml./g. of powder). The combined extracts were centrifuged at 2500 *g* for 15 min. to remove starch and other insoluble material with little loss of enzyme activity. The clear centrifuged solution had from two to six times as much enzyme activity as appeared to be present in the original powder. Even the residue after the citrate extraction had activity as high as, or even higher than, the starting material. Table 1 gives the results of a typical experiment, showing the extraction of chlorophyllase from an acetone-dried powder compared with that from a sample of the same batch of powder after extraction with ethanol and ether.

Partial purification. The enzyme could be precipitated by ammonium sulphate between 35 and 70% saturation, but the recovery was poor. Precipitation with acetone gave better recovery and a bigger increase in the specific activity because more material was rendered insoluble.

Before addition of acetone the solution was cooled to 0° and this temperature was maintained as nearly as possible during precipitation and centrifuging. Acetone (0·9 vol.) was added and, after standing for a few minutes, the precipitate was centrifuged down and taken up in water. This contained about 90% of the protein and less than 10% of the enzyme activity. To the supernatant solution from the first precipitate a further 0·9 vol. of acetone was added. The precipitate was centrifuged down and the supernatant solution discarded. The precipitate was taken up in water and centrifuged to remove a small amount of insoluble material. This fraction contained about 60% of the activity and about 1% of the nitrogen of the solvent-extracted powder. Results for one preparation are given in Table 2. Repeating the acetone precipitation increases the specific activity but so far no useful results have been obtained with adsorbents such as calcium phosphate and Celite. Partly purified preparations have low amylase activity; pectin esterase, phosphatase, amine oxidase and polyphenol oxidase are absent.

Table 1. *Extraction of chlorophyllase*

Powders (3 g.) were soaked in 40 ml. of 0·02 M-sodium citrate solution at room temperature overnight. Crude extracts obtained by squeezing through cloth were centrifuged at 2500 *g* for 15 min. to give clarified extracts.

	Total units in fraction	
	Acetone-dried powder	Solvent-extracted acetone-dried powder
Starting material	61·2	60·0
Crude citrate extract	39·0	202·5
Clarified citrate extract	12·5	197·0
Residue	21·8	72·0

Properties of the enzyme

Stability. Nightshade leaves stored at −10° for 9 months retained their activity. An acetone-dried powder of sugar-beet leaves kept for 6 months at room temperature lost less than 20% of its activity. Solutions of both crude and partly purified enzyme at pH values between 6 and 8 kept at 0° lost about 2% of the activity per day; at 20° 30–50% was lost in 24 hr. The partly purified enzyme was destroyed by 10 min. at 65°; 40% of the activity was lost on heating at 60° for 10 min. It was stable for 1 hr. at 45° in citrate buffer, pH 7, but all activity was lost at this temperature in the presence of 40% acetone.

At pH 3 the enzyme was rapidly inactivated and it would stand only brief exposure to pH 4·5.

On dialysis against distilled water in the cold for 48 hr. up to 60% of the activity was lost; the original activity was not restored by adding dialysate nor by adding boiled enzyme.

Variation of activity with acetone concentration. The presence of a solvent such as acetone is essential for enzyme action. Fig. 3 compares the effect of a range of acetone concentrations on the activity of insoluble and soluble enzyme preparations. The optimum concentration was higher for both types of insoluble preparation than for the partly purified soluble preparation.

Variation of activity with pH. Fig. 4 shows the effect of pH on the activity of soluble and insoluble preparations. Michaelis veronal–acetate buffers were used for the whole range. The pH values of the

Table 2. *Partial purification of chlorophyllase*

	Total units	Activity per g. of dry matter	Activity per g. of N
Solvent-extracted acetone-dried powder (40 g.)	920	23	410
Centrifuged citrate extracts (320 ml.)	2020	—	7 200
Acetone ppt. (0–45%)	205	—	6 500
Acetone ppt. (45–65%)	1056	3600	39 000

buffers were affected both by the chlorophyll solution and by the acetone-dried powder and it was therefore necessary to determine the actual pH of a duplicate portion of each incubated system. There was no change in pH during incubation. For the soluble enzyme the optimum was between 7·5 and 8·0, but with the acetone-dried-powder preparation a higher pH of the suspension was required for maximum activity.

Variation of activity with temperature. The results in Fig. 5 show how temperature affected the enzyme activity of a partly purified preparation. The optimum temperature was between 20° and 30°.

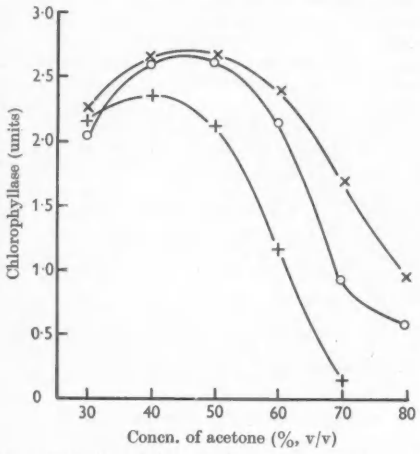

Fig. 3. Variation of chlorophyllase activity with acetone concentration. + Soluble preparation (0·10 mg. of N); ○, acetone-dried powder (200 mg. of dry matter, 13·2 mg. of N); ×, dried-leaf meal (200 mg. of dry matter, 10·6 mg. of N). Incubation, 1 hr.

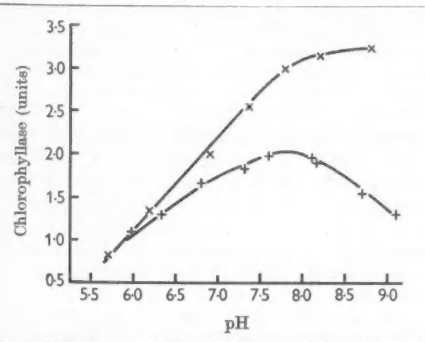

Fig. 4. Variation of chlorophyllase activity with pH. ×, Acetone-dried powder (100 mg. of dry matter, 5·8 mg. of N); +, dialysed soluble preparation (5·1 mg. of dry matter, 0·54 mg. of N). Incubation, 1 hr.

DISCUSSION

The two- to six-fold increase in enzyme activity when chlorophyllase becomes soluble is similar to that for the phenolase of sugar-beet-leaf chloroplasts found by Mayer & Friend (1960) and for aconitase and glutamic acid dehydrogenase of *Lupinus* mitochondria (Estermann, Conn & McLaren 1959). The fact that treatment with ethanol and ether makes subsequent extraction of the enzyme possible suggests that association with lipids, either physical or chemical, may be responsible for the insolubility of the chlorophyllase and also for its being unable to exert maximum activity when *in situ* in the chloroplasts. Ardao & Vennesland (1960) suggest that all the chlorophyllase of the mature spinach leaf may be present in a chlorophyll–lipoprotein complex.

Mayer (1930) found that the enzyme in leaf powders had an optimum pH near 6. This is much lower than was found here, where the activity of an acetone-dried powder increased as the pH of the suspension was raised to almost 9. The pH optimum of the soluble enzyme was between 7·5 and 8, but with a powder a higher pH of the suspension is probably necessary to achieve the optimum pH at the site of enzyme action.

The present findings confirm the need for a high concentration of organic solvent, though the optimum concentration was rather lower (40–50 %

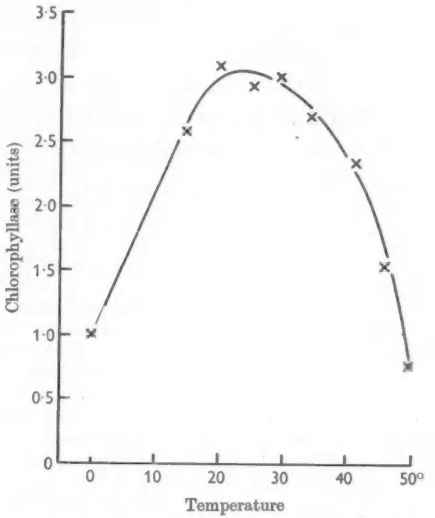

Fig. 5. Variation of chlorophyllase activity with temperature. Soluble preparation (0·10 mg. of N). Incubation, 1 hr.

acetone), than found in earlier investigations. Mayer (1930) used a concentration of 66% for his determinations, but this is definitely inhibitory, though less so for insoluble preparations than for soluble.

The enzyme is remarkably stable before it becomes soluble and the activity of soluble preparations is retained well in the cold. The optimum temperature for enzyme action is much lower than for many enzymes, presumably because the temperature at which the enzyme is inactivated is comparatively low, particularly in the presence of acetone.

The rise in chlorophyllase activity when dark-grown pea seedlings are transferred to light is similar to that found by Hageman & Arnon (1955) for triphosphopyridine nucleotide-linked glyceraldehyde phosphate dehydrogenase. Marcus (1960) induced the formation of triose phosphate dehydrogenase, without the development of chlorophyll, in kidney-bean leaves by brief exposure to red light. Booth (1960) found that a carotene-destroying enzyme system was not normally present in potatoes and carrots, but exposure to light leading to chlorophyll formation in the surface layers also caused the enzyme to appear.

SUMMARY

1. Soluble chlorophyllase preparations were made from sugar-beet leaves, which have the highest activity of all species tested.

2. For the partly purified enzyme the optimum conditions for enzyme action are pH 7·7 at 25° and an acetone concentration of 40%.

3. When dark-grown pea seedlings with very low chlorophyllase activity were transferred to daylight the activity rose in 48 hr. to a level comparable with that of light-grown seedlings.

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Pyridoxamine Phosphate-Oxidase and Pyridoxal Phosphate-Phosphatase Activities in *Escherichia coli*

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Beechey & Happold (1955) first demonstrated the conversion of pyridoxamine phosphate into the aldehyde form by cell-free extracts of *Escherichia coli*. The nature of the product was established by its ability to activate apotryptophanase, an enzyme also present in the aqueous extracts. Optimum conditions for enzyme assay were studied, and simple kinetic studies were carried out by spectrophotometric methods, by Beechey & Happold (1957), who found that *E. coli* extracts catalysed the disappearance of pyridoxal phosphate and that light-absorption at the wavelength characteristic of pyridoxamine phosphate increased during the reaction. The reaction involved thus appeared to be reversible, although only the conversion of pyridoxamine phosphate into the pyridoxal ester required Mg^{2+} ions for maximum activity.

The possibility that more than one enzyme was involved was recognized, but the apparent reversibility of the reaction, and other data, led these workers to assume that a transamination reaction was involved. Unlike Meister, Sober & Tice (1951),

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who presented evidence for transamination between pyridoxamine phosphate and pyruvic acid in extracts of *Clostridium welchii*, Beechey & Happold (1957) were unable to identify amino-group acceptor–donors in their system.

The present paper suggests that the apparent interconversion of pyridoxamine phosphate and pyridoxal phosphate in extracts of *E. coli* is due to the presence of two separate enzymes. These enzymes have been identified as pyridoxamine phosphate oxidase and a pyridoxal phosphate phosphatase. Pogell (1958) has reported the existence of similar enzyme activities in mammalian tissues, also in yeast and *Streptococcus faecalis*.

EXPERIMENTAL

Materials

The preparation and storage of cell-free extracts of acetone–ether-dried *E. coli* were carried out as described by Beechey & Happold (1957). Glutamic–aspartic transaminase was prepared from heart muscle by the method of Cammarata & Cohen (1951), the fraction 'Ppt. WFT-II' being used. An alkaline-phosphatase preparation (Intestinal Phosphatase) was obtained from Armour Laboratories, Chicago, Ill., U.S.A., and a soluble enzyme reagent (Polidase-S), known to contain acid phosphatase, was obtained from Schwartz Laboratories Inc., New York, N.Y., U.S.A. Pyridoxal phosphate and pyridoxamine phosphate were from Hoffmann–La Roche and Co. Ltd., keto acids from British Drug Houses Ltd. and adenosine triphosphate (ATP) from L. Light and Co. Ltd.

Assay of vitamin B_6 compounds and associated enzyme activities

The assay of vitamin B_6 compounds was carried out spectrophotometrically with a Unicam spectrophotometer, model SP. 500. Preliminary experiments were carried out to determine maximum absorption wavelengths for pyridoxamine phosphate and pyridoxal phosphate, also pyridoxal under the conditions in which these compounds were to be assayed.

Composition of standard reaction mixture. For interconversions of vitamin B_6 compounds, reactions were carried out in a total volume of 4·0 ml. The standard composition was: buffer, 1·0 ml.; 0·02 M-$MgSO_4$, 0·5 ml.; concentrated cell extract, approx. 10·0 mg. of protein/ml., 2·0 ml.: vitamin B_6 compound (200 μmg./ml.) 0·5 ml., i.e. approx. 0·4 μmole of pyridoxamine phosphate and pyridoxal phosphate. Water replaced substrate in the blank and replaced $MgSO_4$ solution when pyridoxal phosphate was used as substrate. When the effect of various additions was investigated, cell extract was made up in buffer, and the additions were contained in 1·0 ml. Where necessary, reaction-mixture components were adjusted to the pH of the reaction.

Measurement of the conversion of pyridoxamine phosphate into the aldehyde form. The buffer used was 0·05 M-$NaHCO_3$–Na_2CO_3, pH 10·1 (Delory & King, 1945). Blank, and reaction mixture without substrate, were equilibrated at 37° in 1 cm. quartz cells, the latter being placed in a thermostatically controlled cell-holder. Addition of substrate to the equilibrated reaction system, with stirring, was timed. Extinction values were then read alternatively at 312·5 and 390 mμ, at timed intervals over the period of the reaction. Overall changes in extinction were calculated after extrapolation of readings to zero time.

As the thermostatically regulated cell-holder had positions for only two cells, the complete reaction mixture was used as a blank when the activity of reaction mixture plus addition was of interest. In this case inhibition or activation was related to enzyme activity measured immediately beforehand as described above. When inhibition was observed, readings were made with reaction mixture plus inhibitor in the blank position.

Alternatively, reaction mixtures minus substrate were made up in thin-walled test tubes and equilibrated in a water bath at 37°. Substrate was then added at timed intervals to all but the controls, and at the end of the reaction 1·0 ml. of 2·5 N-NaOH was added to all tubes. Substrate was then added to controls. Pyridoxamine phosphate is stable during incubation under the above-mentioned conditions in the absence of enzyme preparation. Precipitated protein was removed by centrifuging at 10 000 rev./min. for 30 min. (12 000 *g* at the bottom of the tube). The extinction of tube contents was read against the corresponding blank in order to assay the product formed. In later work, the extinction of the control was read against the reaction mixtures in order to assay the substrate utilized. The wavelengths used were the wavelengths of maximum absorption ($\lambda_{max.}$), determined for pyridoxamine phosphate and pyridoxal phosphate in 0·5 N-NaOH, i.e. 307·5 and 390 mμ respectively.

Measurement of the dephosphorylation of pyridoxal phosphate. The same spectrophotometric methods were used as described for the measurement of the conversion of pyridoxamine phosphate into the aldehyde form at pH 10·1. Here, however, product formation was measured by the increase in extinction at 300 mμ, i.e. $\lambda_{max.}$ for pyridoxal at pH 10·1.

Methods used for converting measured changes in extinction into changes in vitamin B_6-compound concentration are described in the Results section. Specific activities of the preparations are expressed in terms of μmoles/30 min./mg. of protein/ml. of reaction mixture.

Isolation and identification of vitamin B_6 compounds. A Dowex-1 formate column, described for the separation of pyridoxine metabolites by Rodwell, Volcani, Ikawa & Snell (1958), was used to isolate vitamin B_6 compounds from reaction mixtures. Deproteinization was by the addition of 100% trichloroacetic acid. Eluate samples were examined spectrophotometrically at two pH values, and vitamin B_6 compounds were detected by the blue colour produced with 2:6-dichloroquinonechloroimide reagent (Snyder & Wender, 1953). Identification was carried out by paper chromatography (Rodwell *et al.* 1958; Fasella & Baglioni, 1956).

Measurements of acid- and alkaline-phosphatase activities

The same composition of reaction mixture was used as for the spectrophotometric assay for interconversion of vitamin B_6 compounds. The protein concentration of *E. coli* extracts used was 2 mg./ml. and that of phosphatase preparations from other sources was usually 1 mg./ml.

E. coli extracts were dialysed overnight against a large volume of the appropriate buffer, before use, to remove traces of inorganic phosphate. In all cases the substrate was disodium phenyl phosphate (0·1 M). Deproteinization was accomplished by addition of 100% (w/v) trichloroacetic acid until no further precipitation of protein occurred, and, after centrifuging to remove protein, 1 ml. of supernatant was used for phosphate assay. The method of Gomori (1941–42) was used to determine inorganic phosphate.

Measurements of tryptophanase activity and indole assay

These were carried out as described by Beechey & Happold (1957).

RESULTS

Conversion of pyridoxamine phosphate into pyridoxal phosphate

Progress of the reaction and product identity. Spectrophotometric data showing the disappearance of pyridoxamine phosphate used as substrate, and the formation of product, are shown in Fig. 1. The absorption spectrum of the product, with peak absorption at 390 mμ (Fig. 2), confirms previous enzymic evidence on the identity of the product as pyridoxal phosphate (Beechey & Happold, 1957). Owing to the high light-absorbency of the cell extracts used, it was not possible to make measurements at wavelengths less than those indicated.

Effect of various compounds, also dialysis, on enzyme activity. In view of the previously reported suggestion that the reaction involved was a transamination, the effect of various potential amino-group acceptors on the activity of cell extracts was investigated. Glyoxylate, shown by Metzler, Ikawa & Snell (1954) to function as an amino-group acceptor in a number of transamination systems, inhibited the above-mentioned reaction when used as the sodium salt at a number of concentrations (Table 1). Dialysis of extracts against $NaHCO_3$–Na_2CO_3 buffer, pH 10·1, decreased their activity by between 41·0 and 42·5%. This activity loss was calculated by comparison with the activity of an extract sample allowed to stand under the

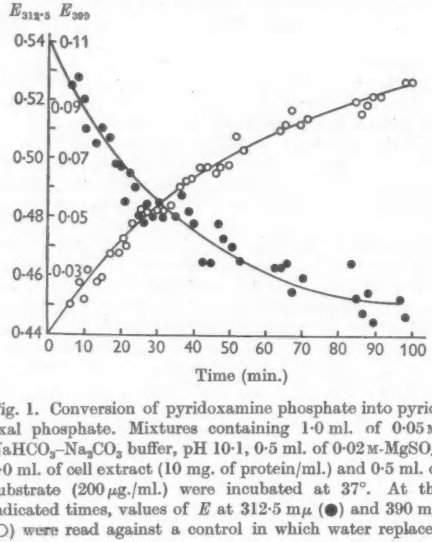

Fig. 1. Conversion of pyridoxamine phosphate into pyridoxal phosphate. Mixtures containing 1·0 ml. of 0·05 M-$NaHCO_3$–Na_2CO_3 buffer, pH 10·1, 0·5 ml. of 0·02 M-$MgSO_4$, 2·0 ml. of cell extract (10 mg. of protein/ml.) and 0·5 ml. of substrate (200 μg./ml.) were incubated at 37°. At the indicated times, values of E at 312·5 mμ (●) and 390 mμ (○) were read against a control in which water replaced substrate.

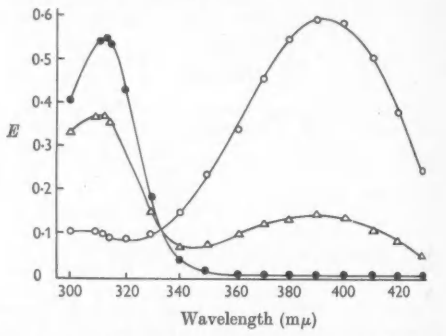

Fig. 2. Absorption spectrum of reaction product with pyridoxamine phosphate as substrate. Reaction conditions were as described in Fig. 1. △, Difference spectrum of product; ●, pyridoxamine phosphate (approx. 25 μg./ml.) incubated under the same conditions but without enzyme; ○, pyridoxal phosphate (approx. 25 μg./ml.). All spectra were plotted after an incubation time of approx. 120 min.

Table 1. *Effect of sodium glyoxylate on the conversion of pyridoxamine phosphate into pyridoxal phosphate*

Reaction mixtures contained: 1·0 ml. of 0·05 M-$NaHCO_3$–Na_2CO_3 buffer, pH 10·1; 0·5 ml. of sodium glyoxylate solution in buffer; 0·5 ml. of 0·02 M-$MgSO_4$; 2·0 ml. of cell extract (10 mg. of protein/ml.); 0·5 ml. of pyridoxamine phosphate (200 μg./ml.; 0·4 μmole). Buffer replaced sodium glyoxylate solution in the control, and water replaced substrate in the blank. At the end of incubation for 30 min. at 37°, 0·5 ml. of 2·5 N-NaOH was added, precipitated protein was removed by centrifuging and E at 390 mμ was read against the blank, in which cell extract was omitted until after the addition of NaOH.

Concn. of sodium glyoxylate (mM)	Increase in E at 390 mμ	Activity (%)
—	0·046	100
0·25	0·037	80
0·63	0·039	85
1·25	0·038	83

same conditions of buffer concentration and pH, but without dialysis; this sample was finally diluted to the same protein concentration as the dialysed preparation. The effect of a number of potential amino-group acceptors on the activity of dialysed cell extracts is shown in Table 2. With such extracts glyoxylate did not cause inhibition. Although the use of boiled cell-extract concentrates suggested that activity could be partially restored to dialysed cell extracts, the results were of little significance. Concentrated diffusate from the extract, observed to have an absorption maximum at 260 mμ at pH 7·0 and to exhibit green fluorescence in u.v. light, did not restore activity. The diffusate was obtained by dialysis of extracts against distilled water and was then concentrated by freeze-drying.

Table 2. *Effect of potential amino-group acceptors on the conversion of pyridoxamine phosphate into pyridoxal phosphate*

Reaction mixture and assay procedure details were as described for Table 1, except that dialysed cell extract was used where appropriate, and incubation was for 90 min.

Additions to dialysed extract (0·02 mM)	Increase in E at 390 mμ	Activity (%)
—	0·036	58
Pyruvate	0·020	35
α-Oxoglutarate	0·003	5
Oxaloacetate	0·008	13
Glyoxylate	0·036	58
Non-dialysed extract (no additions)	0·061	100

Table 3. *Effect of anaerobic conditions on the conversion of pyridoxamine phosphate into pyridoxal phosphate*

The reaction mixture contained in the main arm of Thunberg vessels: 1·0 ml. of 0·05 M-$NaHCO_3$–Na_2CO_3 buffer, pH 10·1; 0·5 ml. of 0·02 M-$MgSO_4$; 2·0 ml. of undialysed cell extract (approx. 10 mg. of protein/ml.). Either 0·5 ml. of water or pyridoxamine phosphate (200 μg./ml.; 0·4 μmole) was contained in the side arm. After repeated evacuation, and flushing with either air or nitrogen, vessels and contents were equilibrated at 37°. Reactions were started by tipping side-arm contents into the main arm. Incubation was for 90 min. Reactions were stopped by the addition of NaOH, and E at 390 mμ was read as described for Table 2.

Conditions of reaction (atmosphere)	Increase in E at 390 mμ	Activity (%)
Air	0·053	100
Nitrogen	0·011	19
Air	0·049	100
Nitrogen	0·015	30
Air	0·062	100
Nitrogen	0·018	29

Nature of the reaction. Since the conversion of pyridoxamine phosphate into the aldehyde form could be partly due to an oxidation process, the effect of anaerobic conditions was investigated (Table 3). At least 70–80 % of the pyridoxal phosphate was formed by an oxidation process. Previous indications suggested that the process might require a diffusible cofactor, but neither riboflavin nor riboflavin phosphate (0·01 mM) restored activity. The antimalarial mepacrine [6-chloro-9-(-4-diethylamino-1-methylbutylamino)-2-methoxyacridine], however, at a concentration of 0·53 mM inhibited the activity of undialysed extracts by about 20 %. Higher mepacrine concentrations could not be employed at pH 10·1 owing to its insolubility. Various flavin-dependent oxidases have been shown to be inhibited by this compound (Hellerman, Lindsay & Bovarnick, 1946; Nygard & Thellwell, 1955; Doisy, Richert & Westerfield, 1955).

Method evolved to calculate changes in substrate and product concentration

In order to correlate observed changes in extinction with changes in substrate and product concentration, certain properties of the respective absorption spectra were found to be of importance. Thus, at pH 10·1, whereas pyridoxamine phosphate does not absorb light at wavelengths above 370 mμ, pyridoxal phosphate absorbs both at 390 mμ ($\lambda_{max.}$) and 312·5 mμ. Thus during the enzymic oxidation of pyridoxamine phosphate to its aldehyde form, any decrease in E observed at 312·5 mμ would be partly masked by an increase due to pyridoxal phosphate formation. General formulae were therefore constructed, relating observed E (1 cm. light path) to the concentration of both pyridoxamine phosphate (Pam.P) and pyridoxal phosphate (Pal.P) in terms of molecular extinction coefficients. During substitution of constants into such formulae, complications due to overlapping of absorption spectra at the wavelengths used are automatically allowed for. The general formulae are:

$$[\text{Pam.P}] = \frac{\epsilon^{390}_{\text{Pal.P}} E^{1\text{ cm.}}_{312\cdot5} - \epsilon^{312\cdot5}_{\text{Pal.P}} E^{1\text{ cm.}}_{390}}{\epsilon^{390}_{\text{Pal.P}} \epsilon^{312\cdot5}_{\text{Pam.P}} - \epsilon^{312\cdot5}_{\text{Pal.P}} \epsilon^{390}_{\text{Pam.P}}}$$

$$[\text{Pal.P}] = \frac{\epsilon^{312\cdot5}_{\text{Pam.P}} E^{1\text{ cm.}}_{390} - \epsilon^{390}_{\text{Pam.P}} E^{1\text{ cm.}}_{312\cdot5}}{\epsilon^{312\cdot5}_{\text{Pam.P}} \epsilon^{390}_{\text{Pal.P}} - \epsilon^{390}_{\text{Pam.P}} \epsilon^{312\cdot5}_{\text{Pal.P}}}.$$

It was found that, at pH 10·1,

$$\epsilon^{312\cdot5}_{\text{Pal.P}} = 1000; \quad \epsilon^{390}_{\text{Pal.P}} = 6000;$$
$$\epsilon^{312\cdot5}_{\text{Pam.P}} = 5700; \quad \epsilon^{390}_{\text{Pam.P}} = 0.$$

Results supporting these values are included in Table 4, lines 1 and 5. Substituting these values in the general formulae,

$$[\text{Pam.P}] = (1\cdot75\, E^{1\text{ cm.}}_{312\cdot5} - 0\cdot29\, E^{1\text{ cm.}}_{390}) \times 10^{-4}\text{ M}$$
$$[\text{Pal.P}] = 1\cdot67\, E^{1\text{ cm.}}_{390} \times 10^{-4}\text{ M}.$$

In order to check the validity of the formulae, mixtures of standard solution of pyridoxamine phosphate and pyridoxal phosphate were prepared at two concentrations. The known concentration values for each component were then compared with values calculated from the values of E observed; good agreement was obtained.

Stoicheiometry of the reaction. By following the progress of the reaction now deduced to be an oxidation of pyridoxamine phosphate, with time, as shown in Fig. 1, it was found that the relationship between the amounts of substrate utilized and product formed was variable. This non-equivalence was particularly marked when extracts of *E. coli* cells grown on a medium including 0·2 % of glucose were used (Fig. 3). A quantitative examination of the results illustrated in Figs. 1 and 3, with the specially derived formulae, suggested a more rapid removal of pyridoxal phosphate from the system when extracts prepared from cells grown on glucose containing media were used. The calculated results

Fig. 3. Conversion of pyridoxamine phosphate into pyridoxal phosphate by extracts of cells grown on a medium containing 0·2 % of glucose. Reaction conditions were as described in Fig. 1. ●, Measured at 312·5 mμ; ○, measured at 390 mμ.

Table 4. *Comparison of known and calculated concentrations of pyridoxamine phosphate and pyridoxal phosphates*

Standard solutions of pyridoxamine phosphate (Pam.P) and pyridoxal phosphate (Pal.P) were prepared, each at 0·12 mM and at one-half of this concentration, in 12·5 M-$NaHCO_3$–Na_2CO_3 buffer, pH 10·1. Mixtures of these solutions were made up as indicated, and values of E (1 cm. light-path) were measured at 312·5 and 390 mμ. Concentration values were calculated as described in the text.

Known concn. of mixture components (0·1 mM)		Content of Pal.P in mixture	$E^{1\,cm.}_{m\mu}$		Calc. concn. of mixture components (0·1 mM)	
Pam.P	Pal.P	(%)	312·5 mμ	390 mμ	Pam.P	Pal.P
1·20	0	0	0·685	0	1·20	0
0·90	0·30	25	0·540	0·180	0·890	0·300
0·60	0·60	50	0·400	0·360	0·595	0·600
0·30	0·90	75	0·265	0·535	0·305	0·890
0	1·20	100	0·120	0·720	0	1·20
0·60	0	0	0·340	0	0·595	0
0·45	0·15	25	0·270	0·090	0·445	0·150
0·30	0·30	50	0·200	0·180	0·280	0·300
0·15	0·45	75	0·135	0·267	0·159	0·445
0	0·60	100	0·070	0·360	0·017	0·600

Table 5. *Apparent stoicheiometric relationships between substrate and product during oxidation of pyridoxamine phosphate*

The reaction mixture is described in the Experimental section of the paper; 5 mg. of protein was present/ml.

Source of data	Reaction time (min.)	Calc. changes in concn. (0·1 mM) Pam.P (a)	Pal.P (b)	Ratio: $\Delta(a)/\Delta(b)$
Fig. 1	0	0	0	—
(Extract 1)	30	0·10	0·09	1·11
	60	0·15	0·13	1·15
	90	0·18	0·16	1·12
Fig. 3	0	0	0	—
(Extract 2)	30	0·19	0·07	2·70
	60	0·24	0·08	3·00

are given in Table 5. From Table 5, the specific oxidase activities of extracts 1 and 2 can be calculated as 2·0 and 3·8 μm-moles of pyridoxamine phosphate/30 min./mg. of protein respectively.

Evidence for the removal of pyridoxal phosphate from reaction systems was also obtained when pyridoxamine phosphate was used as substrate and added apotryptophanase preparations were used to assay pyridoxal phosphate formation. The method used was that described by Beechey & Happold (1957). With either *E. coli* extracts or mammalian glutamic–aspartic-transaminase preparations it was found that an initial rise in the pyridoxal phosphate formation was followed by a rapid fall in the ability of samples to activate the apotryptophanase system.

In early experiments, designed to determine the equilibrium position for the reaction converting pyridoxamine phosphate into pyridoxal phosphate, it was observed that the initial decrease in E at 312·5 mμ was followed after several hours by a slow rise. A simultaneous slow decrease in E at 390 mμ was also observed. These results suggested, in retrospect, the conversion of pyridoxal phosphate into some compound having an absorption at 312·5 mμ similar to, but not identical with, that due to pyridoxamine phosphate.

Conversion of pyridoxal phosphate into free pyridoxal

Establishment of the nature of the reaction. Pyridoxal phosphate is readily hydrolysed to the free base and inorganic phosphate (Peterson, Sober & Meister, 1953), and it appeared likely that the disappearance of this compound from enzymic-reaction systems could be due to enzymic hydrolysis.

In order to distinguish between pyridoxal formation and the production of pyridoxamine phosphate as suggested by the data of Beechey & Happold (1957), the light-absorptions of reaction mixtures with pyridoxal phosphate as substrate were measured at 300, 312·5 and 390 mμ, over a period of approx. 40 min. at 37°. At pH 10·1, the wavelengths quoted characterize the peaks of maximum absorption for pyridoxal, pyridoxamine phosphate and pyridoxal phosphate respectively. The results of one experiment are shown in Fig. 4. Absorption spectrum of the reaction product in a similar experiment is shown in Fig. 5, together with those of pyridoxal and pyridoxal phosphate incubated under the same conditions but in the absence of enzyme preparation. It can be seen that the cell extracts catalysed the breakdown of pyridoxal phosphate to the free base. As the extinction of pyridoxal at 312·5 mμ is appreciable compared with that at 300 mμ, the apparent formation of pyridoxamine phosphate previously reported is thus explained.

Column chromatography of reaction mixtures in which pyridoxal phosphate had been used as substrate and where extracts had been prepared from cells grown on media, with or without added glucose, failed to show any pyridoxamine phosphate or free pyridoxamine. Only pyridoxal was detected in eluate samples.

Effect of various additions. In order to examine the possibility that the dephosphorylation of pyridoxal phosphate might be due to a reversal of the pyridoxal-kinase reaction (Hurwitz, 1953)

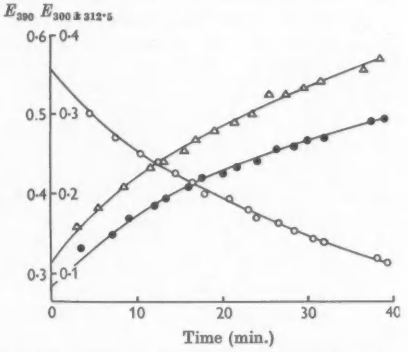

Fig. 4. Conversion of pyridoxal phosphate into free pyridoxal. Reaction conditions were as described in Fig. 1, except that pyridoxal phosphate (approx. 200 μg./ml.) was the substrate, and water replaced $MgSO_4$ solution. ●, Measured at 312·5 mμ; △, measured at 300 mμ; ○, measured at 390 mμ.

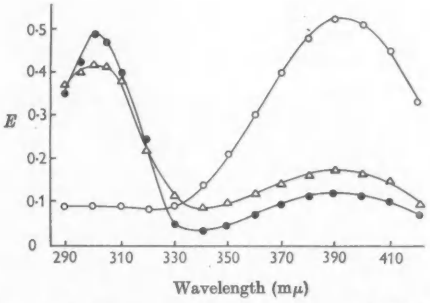

Fig. 5. Absorption spectrum of reaction product with pyridoxal phosphate as substrate. Reaction conditions were as specified in Fig. 4; reaction time, 5 hr. △, Difference spectrum of product; ○, pyridoxal phosphate, approx. 25 μg./ml.; ●, pyridoxal, approx. 25 μg./ml. Both pyridoxal and pyridoxal phosphate were incubated under the same conditions as when pyridoxal phosphate was used as substrate, except that no enzyme preparation was present.

rather than a hydrolysis, the effect of ATP, at a final concentration of 5 mM, on the dephosphorylation of pyridoxal phosphate was investigated. No unequivocal evidence was obtained that ATP affected the rate of dephosphorylation. Spectrophotometric results were complicated by the fact that the *E. coli* extracts appeared to catalyse a decrease in *E*, due to ATP, at 300 mμ, i.e. the wavelength used to assay pyridoxal formation. Under the conditions previously employed for the dephosphorylation of pyridoxal phosphate, no pyridoxalkinase activity could be detected spectrophotometrically with pyridoxal and ATP as substrates, and with *E* at 390 mμ as the criterion of pyridoxal phosphate formation. This confirms the finding of Beechey & Happold (1957), who used enzymic methods for the detection of pyridoxal phosphate.

A study of the effect of Mg^{2+} ions at 2·5 mM on the dephosphorylation of pyridoxal phosphate showed a consistent 8% increase in activity. Ethylenediaminetetra-acetate, however, at a concentration of 0·62 mM, caused no inhibition when non-dialysed cell extract was used.

With either dialysed or non-dialysed *E. coli* preparations, no effect of 0·01 M-NH_4^+ ions on the rate of pyridoxal phosphate utilization could be detected. This contrasts with the apparent 60% increase in rate observed by Pogell (1958), who reported a pyridoxal phosphate phosphatase to be present in mammalian-liver preparations, and suggested the formation of an imide type of intermediate in the presence of NH_4^+ ions.

Phosphatase activities in extracts of Escherichia coli, *and the effect of phosphatase preparations on pyridoxal phosphate.* The fact that pyridoxal phosphate was dephosphorylated by *E. coli* extracts posed the question whether the enzyme involved was one specific for this substrate or possibly an alkaline phosphatase of broad specificity.

With disodium phenyl phosphate as substrate, and by measuring inorganic phosphate formation, cell extracts were found to have little activity at pH 10·1. At lower pH values, however, activity increased to an optimum at pH 5·3. Maximum activity was observed with 0·2 mM-Mg^{2+} ions. It thus appeared that cell extracts contained an acid phosphatase. The respective phosphatase activities at pH 5·3 and pH 10·1 were 12·20 and 0·27 μmoles of phosphate/30 min./mg. of protein.

With an alternative source of acid-phosphatase activity, i.e. Polidase-S, no dephosphorylation of pyridoxal phosphate could be detected at pH 10·1. When an alkaline phosphatase was used, i.e. Intestinal Phosphatase, rapid dephosphorylation of pyridoxal phosphate occurred, even at room temperature. These results suggest that, although *E. coli* extracts contain an acid phosphatase, this enzyme is not responsible for the breakdown of pyridoxal phosphate at pH 10·1, but that an alkaline phosphatase present in smaller amounts is involved. Attempts to demonstrate pyridoxal phosphate-phosphatase activity by assay of inorganic phosphate formed were inconsistent, probably owing to the instability of the substrate under the conditions of the assay procedure.

Method evolved to calculate changes in substrate and product concentration. General formulae relating observed changes in *E* to the respective molar concentrations of the two components were derived for mixtures of pyridoxal phosphate and pyridoxal. Thus:

$$[\text{Pal.P}] = \frac{\epsilon^{300}_{\text{Pal.}} E^{1\text{ cm.}}_{\text{Pal.}} - \epsilon^{390}_{\text{Pal.}} E^{1\text{ cm.}}_{300}}{\epsilon^{300}_{\text{Pal.}} \epsilon^{390}_{\text{Pal.P}} - \epsilon^{390}_{\text{Pal.}} \epsilon^{300}_{\text{Pal.P}}}$$

$$[\text{Pal.}] = \frac{\epsilon^{390}_{\text{Pal.P}} E^{1\text{ cm.}}_{300} - \epsilon^{300}_{\text{Pal.P}} E^{1\text{ cm.}}_{390}}{\epsilon^{300}_{\text{Pal.P}} \epsilon^{390}_{\text{Pal.}} - \epsilon^{300}_{\text{Pal.P}} \epsilon^{390}_{\text{Pal.}}}.$$

It was found that, under the conditions used for enzymic dephosphorylation of pyridoxal phosphate, $\epsilon^{300}_{\text{Pal.}} = 5000$; $\epsilon^{390}_{\text{Pal.}} = 1600$; $\epsilon^{300}_{\text{Pal.P}} = 1080$; $\epsilon^{390}_{\text{Pal.P}} = 6000$. Thus the above formulae may be simplified to:

$$[\text{Pal.P}] = (1{\cdot}77\, E^{1\text{ cm.}}_{390} - 0{\cdot}56\, E^{1\text{ cm.}}_{300}) \times 10^{-4}\text{ M}$$

$$[\text{Pal.}] = (2{\cdot}13\, E^{1\text{ cm.}}_{300} - 0{\cdot}40\, E^{1\text{ cm.}}_{390}) \times 10^{-4}\text{ M}.$$

Applying these relationships to the data illustrated in Fig. 4, the specific activities for the dephosphorylation of pyridoxal phosphate by the cell extract obtained were 10·6 and 9·5 μm-moles of pyridoxal and pyridoxal phosphate respectively/30 min./mg. of protein.

Relative pyridoxamine phosphate-oxidase and pyridoxal phosphate-phosphatase activities

The relative activities of pyridoxamine phosphatase oxidase and pyridoxal phosphatase were determined, with extracts prepared from a number of different batches of *E. coli* cells. The cells were grown with or without glucose. Methods used for the calculation of substrate concentration changes were those described above. In each case, the amount of substrate utilized was used as the criterion of activity measurements (Table 6). The pyridoxal phosphate-phosphatase activity of cells grown in a medium containing glucose is considerably in excess of the pyridoxamine phosphate-oxidase activity.

No pyridoxamine phosphate-phosphatase activity could be detected by the assay of inorganic phosphate production in any of the extracts examined.

DISCUSSION

The experimental measurement of pyridoxamine phosphate-oxidase and pyridoxal phosphate-phosphatase activities is complicated by the presence of

Table 6. *Relative pyridoxamine phosphate-oxidase and pyridoxal phosphate-phosphatase activities*

Reaction mixture composition and procedure were as described for Fig. 1, except that cell-extract concentration was 1·0 mg. of protein/ml. (final concn.), and $MgSO_4$ solution was replaced by water when Pal.P was used as substrate. Phosphatase activity was determined immediately after oxidase activity, with the same sample of cell extract.

Cell sample	Growth conditions for *E. coli*	Relative specific activities* Pam.P oxidase (*a*)	Pal.P phosphatase (*b*)	Ratio: (*b*)/(*a*)
1	Without glucose	0·28	0·20	0·72
2		0·12	0·17	1·42
3	With 0·2% of glucose	0·48	0·98	2·05
4		0·20	0·79	3·95

* 10^{-2} μmole/30 min./mg. of protein/ml. of reaction mixture.

both enzymes, in variable proportions, in the cell extracts used. If enzymic or chemical methods are used for the assay of pyridoxal phosphate when pyridoxamine phosphate is used as substrate, then the possible simultaneous breakdown of pyridoxal phosphate is difficult to allow for. On the other hand, spectrophotometric methods allow the precise determination of stoicheiometric relationships between substrates and products. Spectrophotometric methods for the simultaneous determination of pyridoxamine phosphate, pyridoxal phosphate and pyridoxal concentrations in reaction mixtures are at present under development in these laboratories. Such methods allow exact measurement of pyridoxamine phosphate-oxidase activities even in the presence of pyridoxal phosphate phosphatase. The relative activities of pyridoxamine phosphate oxidase and pyridoxal phosphate phosphatase in biological systems is of intrinsic interest. Although Wada *et al.* (1959) claim that phosphatase activity towards pyridoxal phosphate can be removed from crude extracts of liver by precipitation at pH 5·0, Pogell (1958) was unable to remove this activity completely by such treatment.

In the present report, no explanation is given for the decrease in pyridoxamine phosphate-oxidase activity during dialysis of *Escherichia coli* extracts. In view of the linking of oxidase and pyridoxal phosphate-phosphatase activities, it is possible that the loss of some factor affecting the latter is responsible.

It has been reported that pyridoxamine phosphate is the predominating member of the vitamin B_6 group present in the cell (Rabinowitz & Snell, 1947; McNutt & Snell, 1948), and the possible significance of the enzymic conversion of pyridoxamine phosphate into pyridoxal phosphate in the control of amino acid metabolism has been pointed out (Beechey & Happold, 1957; Pogell, 1958). Pyridoxamine phosphate-oxidase activity has been shown to be responsible for the indirect activation of tyrosine decarboxylase (Pogell, 1958), and is now thought to effect the observed activation by pyridoxamine phosphate of tryptophanase (Beechey & Happold, 1957) and kynureninase (Saran, 1958). The role played by pyridoxal phosphate in a large number of enzyme reactions has recently been reviewed by Snell (1958). The inhibition of pyridoxamine phosphate oxidase by α-oxo acids commonly involved in transamination reactions may be part of the mechanism used by cells for metabolic control. Of similar significance would be those factors affecting the activity of pyridoxal phosphate phosphatase. Both Pogell (1958) and Wada *et al.* (1959) have detected phosphatase activities for pyridoxal phosphate in rabbit-liver preparations. In the control of amino acid metabolism by coenzyme destruction, the strength of binding between pyridoxal phosphate and the respective apoenzymes would also be of importance. A number of pyridoxal phosphate-dependent enzymes lose their activity on aging under various conditions but can be reactivated by the addition of synthetic coenzyme (Kallio, 1951; Azarkh & Gladkova, 1952; Happold & Struyvenberg, 1954). The disappearance of bacterial lysine decarboxylase after induction (Mandelstam, 1954) also implicates coenzyme destruction (Pardee, 1958). If local intracellular control of either or both of the oxidase and phosphate enzymes dealt with here is possible, then differential regulation of individual enzymes or groups of enzymes involved in amino acid metabolism would occur. Horiuchi (1959) has recently reported that *Escherichia coli* cells grown on a phosphate-deficient medium exhibit an alkaline-phosphatase activity approximately 100 times as great as that of cells grown on a complete medium. In view of the fact that alkaline phosphatase rapidly destroys pyridoxal phosphate, these findings suggest that the amino acid economy of cells grown on deficient media may be affected by the inactivation of enzymes responsible for amino acid breakdown.

SUMMARY

1. Evidence is presented for the presence of pyridoxamine phosphate oxidase and a pyridoxal phosphate phosphatase in aqueous extracts of *Escherichia coli*.

2. Spectrophotometric methods have been developed for the precise measurement of substrate and product concentrations in both enzyme systems. Specific activities of the order of 10^{-2} μ-mole/30 min./mg. of protein/ml. of reaction mixture are reported.

3. Pyridoxamine phosphate oxidase was inhibited by dialysis, and further inhibition was caused by pyruvate, α-oxoglutarate and oxaloacetate at a concentration of 0·02 mM. Inhibition of activity by mepacrine suggests that a flavin coenzyme is involved.

4. Pyridoxal phosphate phosphatase was also found to be highly active in mammalian 'alkaline'-phosphatase preparations. The *Escherichia coli* extracts used exhibited high 'acid'-phosphatase and low 'alkaline'-phosphatase activities when disodium phenyl phosphate was used as substrate. No dephosphorylation of pyridoxamine phosphate could be detected.

5. The presence of glucose in *Escherichia coli* growth medium increased the relative amount of phosphatase compared with oxidase activity.

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The Sedimentation Characteristics of Deoxyribonucleic Acid from Human Tissues

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Polli & Shooter (1958) have presented the data obtained from sedimentation experiments with preparations of deoxyribonucleic acid from normal human spleen and leucocytes and from leucocytes of patients with chronic myeloid and lymphatic leukaemia. It was found that the average sedimentation coefficients at infinite dilution for preparations from leucocytes of lymphatic leukaemia were higher than those observed for preparations from normal leucocytes. Different preparations

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from these two last-named sources gave reproducible results, whereas the average sedimentation coefficients at infinite dilution for the preparations from leucocytes of myeloid leukaemia exhibited a much greater scatter, ranging from 27s, the value observed for deoxyribonucleic acid from normal leucocytes, to 22·8s. Another group of 23 preparations has now been studied in an attempt to confirm and define more precisely these differences in sedimentation characteristics.

Since it has been shown that the presence of small amounts of residual protein in preparations of deoxyribonucleic acid from calf thymus can markedly affect the distribution curves of sedimentation coefficients (Butler, Phillips & Shooter, 1957), the amount and composition of the residual protein in some of the present preparations have been determined. Attempts have also been made to disrupt any protein-linked aggregates which might be present in the preparations, by treating them with proteolytic enzymes.

EXPERIMENTAL

Blood specimens from leukaemic patients were collected by exchange transfusion and the leucocytes isolated in the manner described by Polli (1951). The sample of normal leucocytes was obtained from the pooled blood of four donors. Placentae were obtained during Caesarean operations and were immediately frozen in solid CO_2: in all four cases studied the tissue can be regarded as normal. The spleen samples were also frozen quickly in solid CO_2 after removal from the patient. Deoxyribonucleic acid (DNA) was prepared from these cells and tissues by the detergent method of Kay, Simmons & Dounce (1952) as modified by Chargaff (1955). Fibrous tissue was removed from the homogenates of placenta and spleen samples by filtration through gauze during the first three stages of washing with 0·14 M-NaCl.

The samples of DNA were dissolved in 0·2 M-NaCl–0·025 M-KH_2PO_4–0·013 M-NaOH, pH 7·5. The sedimentation experiments were performed in a Spinco model E ultracentrifuge fitted with the u.v.-light-absorption optical system. For six of the samples experiments were performed at four concentrations in the range 0·008–0·001% of DNA and curves giving the distribution of sedimentation coefficients were calculated in the manner described by Shooter & Butler (1956). The concentration dependence of the sedimentation coefficients was the same for all six samples, the average sedimentation coefficient at infinite dilution being 1·0–1·2s greater than the average sedimentation coefficient observed at a concentration 0·001–0·0015% of DNA. Experiments with the remaining samples were therefore performed at only one concentration in the range 0·001–0·0015%. The average sedimentation coefficients quoted in the tables are the values at the 50% concentration point on the distribution curves. These values are about 10% less than the weight-average sedimentation coefficients (Butler, Laurence, Robins & Shooter, 1959).

To determine the effect of proteolytic enzymes on the preparations trypsin (Armour: crystalline), and chymotrypsin (L. Light and Co. Ltd.), ficin and papain (Sigma Chemical Co.: crystalline) were added to solutions of DNA in 0·2 M-NaCl pH 7·5, and the sedimentation characteristics were determined after storing for 3–16 days at 4° and then for a further 3–6 hr. at room temperature. In each case a weight of enzyme was added equal to the weight of DNA present in the solution.

The amino acid composition of the residual protein in the preparations was determined in the manner described by Phillips & Johns (1959). The protein content of the preparations quoted in Table 1 represents the sum of the amounts of amino acids which have been determined; no allowance has been made for tryptophan, which is destroyed during the acid hydrolysis, or for glycine, since most of this amino acid observed in the chromatograms is the product of the degradation of adenine and guanine.

RESULTS

The information obtained from the analysis of the residual protein present in eight of the DNA preparations is given in Table 1. It may be noted that the residual proteins in these preparations contain greater amounts of aspartic acid and glutamic acid than of the basic amino acids lysine and arginine. In this respect the analyses resemble those reported for the residual protein in preparations of DNA from calf thymus (Butler *et al.* 1957) and from mouse and rat spleen and liver (Kirby, 1959). The amount and composition of the protein in the preparations from human cells and tissues vary from sample to sample and there is no obvious correlation with the source of the DNA, i.e. there is no evidence that the residual protein represents some part of the nucleoprotein complex characteristic of a particular cell or tissue. Solutions of all the eight preparations of DNA mentioned in Table 1 have been treated with ficin, papain, trypsin and chymotrypsin and in all cases it has been found that the distribution curves of sedimentation coefficients of control and enzyme-treated solutions did not differ by more than ±0·5s, a variation well within experimental error. With some of the preparations of DNA from calf thymus investigated by Butler *et al.* (1957) it was found that treatment with proteolytic enzyme did not affect the average sedimentation coefficient, but the presence of high-molecular-weight, polydisperse aggregates of DNA cross-linked by protein could be deduced from the observed increase in the amount of u.v.-absorbing material sedimenting in the range 10–50s, which was produced by the enzyme treatment. No evidence of this type of effect was found with any of the preparations of DNA used in the present work.

The average sedimentation coefficients for all the preparations investigated are presented in Table 2. In all cases the distribution curves covered a range from 10 to 50s and no significant differences in the shapes of the curves were observed.

Table 1. *Amino acid composition of the residual protein of preparations of deoxyribonucleic acid*

Results are expressed as moles of amino acids/100 moles of all amino acids found. Arginine and histidine were not assayed in samples E, F and G. Cystine, methionine and hydroxyproline were not seen in any hydrolysate: glycine, tryptophan and amide were not determined.

DNA preparation	Leucocytes of myeloid leukaemia				Leucocytes of lymphatic leukaemia		Spleen	Placenta
	E	*F*	*G*	*D*	*B*	*C*	*H*	*A*
Alanine	6·3	6·7	6·3	8·1	7·2	6·9	6·3	8·0
Aspartic acid + Glutamic acid	25·8	9·7 + 9·7	10·7 + 10·7	10·5 + 16·1	11·5 + 13·6	25·5	20·6	10·4 + 16
Valine + Leucines	26·4	29	28	6·5 + 17·7	27·4	8·3 + 15·9	26·2	6·4 + 17
Phenylalanine	9·4	12	12·5	7·3	6·5	5·5	7·5	7·2
Tyrosine	0	0	0	+	0	0	0	+
Serine	14·5	14·3	12	8·1	9·3	10·3	8·7	8
Threonine	6·9	10	8·2	5·6	4·6	4·8	11·2	5·6
Proline	6·9	8	10·7	3·2	5·8	4·8	6·9	3·2
Lysine	3·8	0	0	5·6	3·6	10·3	6·9	5·6
Arginine	—	—	—	8·9	5·1	5·5	3·7	8
Histidine	—	—	—	2·4	5·1	2·1	1·9	4
Total protein (as % of DNA)	0·52	0·29	0·67	0·85	0·63	0·99	0·75	0·99
Average sedimentation coefficient (s)	26·8	27	25·6	23·6	27·6	30	26	26

Table 2. *Average sedimentation coefficients for solutions of deoxyribonucleic acid*

Sedimentation experiments were performed with solutions of DNA in 0·2 M-NaCl, pH 7·5, at concentrations in the range 0·001–0·0015 %. Figures in parentheses are taken from Polli & Shooter (1958) and have been corrected to a DNA concentration of 0·0015 %. Preparations which have been analysed for residual protein content are indicated by the same letter as was used in Table 1.

Source	Clinical condition	Average sedimentation coefficient (s)
Placenta	Normal	26·0 *A*, 25·0, 27·0, 26·2
Leucocytes	Normal	26·0 (26·0, 25·8)
	Lymphatic leukaemia	27·6 *B*, 30·0 *C*, 28·3, (29·8, 28·5, 27·4)
	Myeloid leukaemia (untreated patients)	23·1, 23·6 *D*, 27·0, (21·8, 21·8)
	Myeloid leukaemia:	
	Before treatment with busulphan	(26·0)
	After treatment with busulphan	(23·5)
	Myeloid leukaemia:	
	First blood sample	26·8 *E*
	Second blood sample (7 days after first sample)	27·0 *F*
	Third blood sample (after treatment with busulphan; 10 days after second sample)	25·6 *G*
	Myeloid leukaemia treated with busulphan	26·2
	Acute leukaemia	24·6, 24·0, 25·4
Spleen	Normal	(25·2, 25·8)
	Banti's disease	23·0
	Banti's disease, two preparations from the same tissue	23·1, 22·2
	Hereditary spherocytosis	25·0
	Myeloid leukaemia	26·0 *H*
	Brill–Simmers disease	(28·8)

DISCUSSION

It is evident from the results given in Table 2 that the preparations of DNA that have been studied exhibit quite marked differences in sedimentation characteristics. The major problem is to determine whether these differences reflect real variations in the degree of polymerization of the DNA (cf. Butler *et al.* 1959) or whether they arise from cross-linking of the DNA particles to different extents by the residual protein in the preparations. The attempts that have been made to degrade such aggregates which might be present in our preparations, by treatment with four different proteolytic enzymes, have all proved fruitless. It may also be noted (Table 1) that there is no correlation between total amount of residual protein and the average sedimentation coefficient. It would therefore seem, on the basis of these negative results, that the DNA preparations do not

contain detectable amounts (< 5%) of aggregates cross-linked by protein and hence that the protein molecules or polypeptides must be bound along the lengths of single DNA particles. The amount of protein present is so small that if it is bound in this way it would have an insignificant effect on the sedimentation characteristics of the DNA. We may assume therefore that the differences observed reflect real differences in the degree of polymerization of the DNA.

The average sedimentation coefficients observed for the new preparations of DNA from leucocytes of lymphatic leukaemia and from normal leucocytes agree well with those reported by Polli & Shooter (1958). Preparations of DNA from placenta give distribution curves of sedimentation coefficients very similar to those found for DNA from normal leucocytes. Taking the results for the preparations from placenta and normal leucocytes as characteristic of normal tissues and cells, it is found that the average sedimentation coefficients for the preparations from the leucocytes of lymphatic leukaemia are significantly higher than normal ($P < 0{\cdot}01$). With the molecular-weight determinations of Butler *et al.* (1959) as a basis it can be calculated that DNA from leucocytes of lymphatic leukaemia has a mean molecular weight about 30% higher than normal.

In agreement with the results reported by Polli & Shooter (1958) it has been found that the average sedimentation coefficients for different preparations of DNA from leucocytes of myeloid leukaemia vary considerably. Four of the preparations gave results indistinguishable from the normal and three had lower average sedimentation coefficients. A preparation of DNA from the spleen of a patient with myeloid leukaemia also gave a result similar to the normal. In the one set of preparations available no evidence of any change in the characteristics of DNA obtained from leucocytes of one patient was observed in 7 days (Table 2). After treatment with Myleran (busulphan) the DNA obtained from the leucocytes of the same patient showed a slight decrease of the average coefficient. One further preparation from leucocytes of a patient treated with busulphan has been studied but this did not differ from the normal. No further clinical or histological evidence can be cited which could account for the variations found with this group of preparations from leucocytes of myeloid leukaemia.

The three preparations from the leucocytes of patients with acute leukaemia are very similar, the average sedimentation coefficients being lower than normal. This is too small a group to decide whether the results are characteristic of the disease.

The data obtained for the three preparations of DNA from spleen are of some interest since these diseases would not be classified as malignant. The average sedimentation coefficients for the two preparations from spleen associated with Banti's disease are considerably lower than normal, the decrease in mean molecular weight being about 30%. The preparation from the spleen of the patient with hereditary spherocytosis is close to normal.

The results of the present work confirm the observations of Polli & Shooter (1958) and demonstrate that the physical properties of preparations of DNA from diseased human tissues and cells may be very different from preparations from normal sources, the mean molecular weights of the preparations varying by as much as ±30%. We cannot be sure, however, that the DNA prepared is identical with the DNA in the living cell; degradation may occur during preparation, particularly if there is an abnormally high concentration of deoxyribonuclease in the diseased cells. To find out more about the origin and significance of the variations observed requires detailed studies of the DNA synthetic and degradative mechanisms of the cells.

SUMMARY

1. Distribution curves of sedimentation coefficients at a concentration 0·001–0·0015% have been determined for a group of preparations of deoxyribonucleic acid from normal and diseased human tissues and cells.

2. Preparations from normal sources gave average sedimentation coefficients of 25–27s; all preparations from leucocytes of lymphatic leukaemia were found to have higher than normal sedimentation coefficients, whereas in preparations from leucocytes of myeloid leukaemia the results were the same as, or less than, normal.

3. Sedimentation coefficients lower than normal have been observed with preparations from spleens associated with Banti's disease.

4. Eight of the preparations have been analysed to determine the amount and composition of the residual protein. There appears to be no correlation between these results and the source of the preparation of deoxyribonucleic acid or its sedimentation characteristics.

5. The variation in sedimentation behaviour observed between different samples does not appear to be due to the presence of varying amounts of protein cross-linked aggregates, since treatment with any of the enzymes trypsin, chymotrypsin, ficin or papain has no detectable effect.

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Metabolism of Polycyclic Compounds

18. THE SECRETION OF METABOLITES OF NAPHTHALENE, 1:2-DIHYDRONAPHTHALENE AND 1:2-EPOXY-1:2:3:4-TETRAHYDRONAPHTHALENE IN RAT BILE*

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A metabolite of sulphobromophthalein has been detected in the bile of rats (Combes, 1959) and of human subjects (Grodsky, Carbone & Fanska, 1959), which gives rise to glycine and glutamic acid on acid hydrolysis. Grodsky *et al.* (1959) suggested that the metabolite is a glutathione conjugate. In a study of the metabolism of naphthalene, 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene by rat-liver slices (Booth, Boyland & Sims, 1960*b*; Booth, Boyland, Sato & Sims, 1960*a*), it was found that these compounds are converted into glutathione conjugates, which yield the corresponding cysteine conjugates on incubation with rat-kidney homogenates. The present work shows that the bile of rats treated with either naphthalene, 1:2-dihydronaphthalene or 1:2-epoxy-1:2:3:4-tetrahydronaphthalene contain conjugates of glutathione, cysteinyl glycine, cysteine and *N*-acetylcysteine in addition to other metabolites previously reported as urinary excretion products.

EXPERIMENTAL

Materials. 1:2-Dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene were prepared as described by Boyland & Sims (1960). *S*-(1-Naphthyl)glutathione, *S*-(1-naphthyl)-L-cysteine and 1-naphthylmercapturic acid were prepared by the methods of Booth *et al.* (1960*b*). A product which appeared to be mainly *S*-(1-naphthyl)-L-cysteinylglycine was obtained by boiling *S*-(1-naphthyl)-glutathione (100 mg.) with water (150 ml.) under reflux for 48 hr. The solid obtained on evaporation of the water was extracted with saturated aq. $NaHCO_3$ and the extract acidified with acetic acid to yield a light-brown powder (25 mg.). On paper chromatograms developed with solvent 1 described below, the solid yielded one large and two small spots, the latter being indistinguishable from *S*-(1-naphthyl)glutathione and *S*-(1-naphthyl)-L-cysteine. The large spot was eluted from chromatograms and treated by the methods described below to yield compounds indistinguishable from *S*-(1-naphthyl)-L-cysteine and glycine after hydrolysis with HBr and from alanine and glycine after hydrogenolysis with Raney nickel followed by acid hydrolysis. No other amino acids were detected. A similar result was obtained when *S*-(1-naphthyl)glutathione was heated with N-H_2SO_4 at 100° for 1 hr.

S-(1:2:3:4-Tetrahydro-2-hydroxy-1-naphthyl)glutathione was prepared by the method of Booth *et al.* (1960*a*) and *S*-(1:2:3:4-tetrahydro-2-hydroxy-1-naphthyl)-L-cysteine and *N*-acetyl-*S*-(1:2:3:4-tetrahydro-2-hydroxy-1-naphthyl)-L-cysteine by the methods of Boyland & Sims (1960). A preparation which appeared to be a mixture of the stereoisomeric forms of *S*-(1:2:3:4-tetrahydro-2-hydroxy-1-naphthyl)-L-cysteinylglycine was obtained as a gum when L-cysteinylglycine was treated with 1:2-epoxy-1:2:3:4-tetrahydronaphthalene under the conditions used by Booth *et al.* (1960*a*) for the reaction of the epoxide with glutathione. The gum formed one spot on paper chromatograms, and

* Part 17: Booth, Boyland, Sato & Sims (1960*a*).

this gave positive reactions with the $K_2Cr_2O_7$–$AgNO_3$ and ninhydrin reagents described below, and yielded glycine, cysteine (and cystine) and 2-tetralone [detected by the test of Straus & Rohrbacher (1921)] after acid-hydrolysis, and glycine, alanine and 2-naphthol after hydrogenolysis with Raney nickel (followed by acid-hydrolysis of the dipeptide thus obtained). When the epoxide was treated with the products obtained by boiling glutathione with water for 24 hr., compounds were detected on paper chromatograms indistinguishable from the glutathione and cysteinylglycine derivatives described above and a third product, which gave a positive reaction with the $K_2Cr_2O_7$–$AgNO_3$ reagent but no ninhydrin reaction and is probably a derivative of the diketopiperazine which Hopkins (1929) had shown to be a reaction product of glutathione with boiling water.

Other naphthalene metabolites were isolated from the urine of rabbits dosed with naphthalene as described by Boyland & Sims (1957, 1958) and Sims (1959) and other metabolites of 1:2-dihydronaphthalene were isolated from the urine of rabbits dosed with 1:2-dihydronaphthalene as described by Boyland & Sims (1960).

Paper chromatography. Except where stated, paper chromatography was carried out by downward development for 18 hr. on Whatman no. 1 paper in: 1, butanol–propan-1-ol–aq. 2N-NH_3 soln. (2:1:1, by vol.); 2, butanol–acetic acid–water (2:1:1, by vol.); or 3, 0·1N-NH_3 (upward development for 8 hr.) [this solvent was used in the identification of 1- and 2-naphthol (Sims, 1959)]. The dried chromatograms were examined under u.v. light and sprayed with (i), freshly diazotized *p*-nitroaniline (0·02% in 0·1N-HCl) followed by aq. 10% Na_2CO_3; (ii), 0·1M-$K_2Cr_2O_7$–acetic acid (1:1, v/v) followed by 0·1M-$AgNO_3$ (Knight & Young, 1958); or (iii), aq. 2% (w/v) $NaIO_4$ followed after 30 min. by Schiff's reagent (Brookes & Young, 1956). Other chromatograms were dipped in a solution of ninhydrin in acetone (0·2%) and heated to 70° in an oven for 10 min. In the detection of naphthalene metabolites, chromatograms were also sprayed with 2N-HCl, heated to 70° for 10 min. and sprayed with reagent (i) above.

The presence or absence of the various metabolites in the bile of treated animals was determined by a direct comparison with authentic materials on paper chromatograms wherever possible and by their reactions to the reagents described above. Many of these reactions have been described (Boyland & Sims, 1958, 1960; Sims, 1959).

The amino acids obtained after the acid-hydrolysis of some of the metabolites as described below were characterized on paper chromatograms both by direct comparison with authentic materials with solvent 2 and, after conversion into 2:4-dinitrophenyl derivatives (Sanger, 1945), by comparison with authentic derivatives with the solvents and conditions of Phillips (1958). Glycine and taurine were also detected on chromatograms by the *o*-phthalaldehyde reagent of Curzon & Giltrow (1954).

Establishment of biliary fistulae in rats. Tributaries from the main lobes of the liver join to form the common bile duct which opens into the anterior wall of the duodenum about 3 cm. from the pylorus. The pancreatic ducts drain into the lower one-third of the common bile duct and therefore the upper part of the duct must be cannulated in order to obtain uncontaminated bile.

Under ether anaesthesia an upper right paramedian incision was made and the bile duct displayed by traction on the duodenum. The duct was ligated at its midpoint and then opened just above the ligature, and polythene tubing (0·35 mm. diameter) was introduced by using binocular lenses. The end of the tube was bevelled to facilitate its introduction. (Nylon tubing of equal bore was unsatisfactory because it kinked.) The tube was pushed up the duct to the junction of the lowest tributaries when the flow of bile stopped: it was then withdrawn 0·5 cm. and an atraumatic suture placed around the duct just above the site of cannulation. The suture was tied but not cut. A stab wound was made through the abdominal muscles 1·5 cm. from the right margin of the wound with a needle, which was pushed into the end of the tube and withdrawn, carrying the tube into the subcutaneous tissues. The atraumatic suture then picked up a fold of peritoneum just above the emerging tube and thus anchored it flush with the abdominal wall. A second suture fixed the tube to the muscles in the subcutaneous space and the tube was passed along a hollow needle of suitable calibre to emerge in the midline at the nape of the neck, so that the rat cannot bite the tube. Bile was collected in a glass saddle-shaped container (Van Zyl, 1958) held in position by encircling strips of Elastoplast.

The rats with biliary fistulae were given solutions of naphthalene (75 mg.), 1:2-dihydronaphthalene (50 mg.) or 1:2-epoxy-1:2:3:4-tetrahydronaphthalene (25 mg.) in arachis oil (0·5 ml.) by intraperitoneal injection on the day after the operations and on each successive day until the animals were ill. Bile was collected daily and stored at 0°. The rats each produced about 25 ml. of bile every day and usually lived for about 5 days after the operations.

Treatment of bile. The biles collected from each animal were pooled and treated separately. A direct examination of the bile on paper chromatograms showed that the normal biliary constituents had little effect on the R_F values of the metabolites. Usually, however, the bile was diluted with 3 vol. of water and the solution acidified to pH 4 with acetic acid and activated charcoal (1 g. for every 25 ml. of undiluted bile) added with stirring. The charcoal was filtered off and washed with water (250 ml.) and the absorbed material eluted by passing first 500 ml. of methanol containing 5% (v/v) of aq. NH_3 soln. (sp.gr. 0·88) and then methanol containing 5% of benzene through the filter. The combined eluates were evaporated to dryness under reduced pressure, and the residue (which contained bile salts) was examined on paper chromatograms.

The examination of the bile for amino acid conjugates was carried out separately. The materials obtained with the charcoal procedure described above were applied along the base-lines of chromatograms [one sheet of Whatman no. 1 paper (46 cm. × 57 cm.) was used for the material obtained from each 20 ml. of bile] which were developed with solvent 1. With naphthalene, the zones on the dried chromatograms containing the conjugates were located as dark absorbent regions in u.v. light, and on test strips cut from the main chromatograms as regions giving blue colours with diazotized *p*-nitroaniline and Na_2CO_3 and positive reactions with the $K_2Cr_2O_7$–$AgNO_3$ reagent. Except for the *N*-acetylcysteine conjugate, positive ninhydrin reactions were also obtained. With 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene, the zones were located with the $K_2Cr_2O_7$–$AgNO_3$ reagent, applied to test strips cut from the main chromatograms.

The zones were cut from the chromatograms, and the conjugates were eluted with either aq. 2N-NH_3 or with methanol containing 5% (v/v) of aq. NH_3 soln. (sp.gr. 0·88). The eluates were evaporated to small volumes under reduced pressure to give solutions of the conjugates under investigation, which in most cases also probably contained naturally occurring taurine and glycine conjugates. Grodski *et al.* (1959) found that Whatman 3MM chromatography paper contained substances which gave rise to glycine and glutamic acid on acid hydrolysis. In the present work it was found that the amounts of amino acids arising in this way were negligible as compared with those arising from the hydrolysis of the metabolites. On the other hand, experiments in which bile from untreated animals was examined on chromatograms under the above conditions showed that contamination from biliary glycine and taurine conjugates could easily arise. With the conjugates arising from naphthalene, a third source of contamination was present, namely the conjugates themselves which break down spontaneously during the working-up procedures to give small amounts of peptides which yield glycine and sometimes glutamic acid on acid hydrolysis (a similar but faster breakdown of the metabolites in the presence of mineral acid is described later). This possibility of contamination was also present, but to a much lesser extent, with the amino acid conjugates of 1:2-dihydronaphthalene, since this hydrocarbon is converted into naphthalene amino acid conjugates in the body to some extent.

The solutions containing the amino acid conjugates of naphthalene were each divided into two portions, one of which was applied to the base-lines of chromatograms which were developed with solvent 2. The other portions were acidified with HCl and the solutions applied to the base-lines of chromatograms which were developed with solvent 1. The conjugates were all related to *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine, which yields 1-naphthylmercapturic acid on acidification (Boyland & Sims, 1958) and all gave similar acid decomposition products which had R_F values different from those of the original conjugates. Normal biliary constituents would not be expected to be affected by this mild treatment, and their R_F values would remain the same. The new conjugates were located on the chromatograms by their characteristic orange fluorescence under u.v. light. The elution of the appropriate zones of the chromatograms with aq. 2N-NH_3 soln. afforded solutions of the conjugates, or of their acid decomposition products, which were evaporated under reduced pressure to yield gums which were treated as described below.

The bile from animals treated with 1:2-dihydronaphthalene contained, besides the amino acid conjugates of this hydrocarbon, detectable amounts of amino acid conjugates which were indistinguishable from those formed from naphthalene as described above. The corresponding amino acid conjugates of the two hydrocarbons were difficult to separate from each other on chromatograms, so that the solutions of the conjugates of 1:2-dihydronaphthalene obtained as described above contained small amounts of those of naphthalene. If the solutions were acidified with HCl and chromatographed in solvent 1, however, the amino acid conjugates of naphthalene were decomposed, in the manner indicated above, to give products of different R_F values from the corresponding conjugates of 1:2-dihydronaphthalene, which are unaffected by cold dilute acid. The amino acid conjugates of 1:2-dihydronaphthalene, now free from the corresponding conjugates of naphthalene, were eluted from the appropriate zones on the chromatograms with aq. 2N-NH_3 soln., and the solutions applied to the base-lines of chromatograms which were developed in solvent 2. The conjugates were located and eluted as before and the solutions evaporated to yield gums which were treated as described below.

The solution obtained from the initial fractionation of the bile of animals treated with 1:2-epoxy-1:2:3:4-tetrahydronaphthalene, which did not contain amino acid conjugates of naphthalene, were applied to the base-lines of chromatograms which were developed with solvent 2. The conjugates were located and eluted, and the solutions evaporated as before to yield gums which were treated as described below.

With the above techniques, a number of gummy products (in mg. quantities) were obtained, which gave single spots on paper chromatograms with the $K_2Cr_2O_7$–$AgNO_3$ reagent and (except for the mercapturic acids) with ninhydrin. From the bile of animals treated with naphthalene six pairs of products (from normal and acidified fractions) were obtained, whereas bile from animals treated with 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene each yielded five products.

Examination of the amino acid conjugates obtained from naphthalene. Small portions of the conjugates were each dissolved in a few drops of water and the solutions acidified with HCl. The products were examined on paper chromatograms with solvents 1 and 3.

Portions of the conjugates were separately dissolved in methanol (5 ml.) and heated under reflux with Raney nickel (W2 grade, 100 mg.) for 2 hr. The mixtures were filtered and the filtrates diluted with water (25 ml.) and extracted with ether (2 × 25 ml.). The ethereal extracts were evaporated and the residues examined for naphthols with solvent 3. The aqueous phases were evaporated and the residues heated with HBr (sp.gr. 1·7) at 100° for 4 hr. The solutions were evaporated and the residues examined for amino acids, both directly with solvent 2 and after treatment with 2:4-dinitrofluorobenzene as indicated above.

Other portions of the conjugates were heated with HBr (sp.gr. 1·7) for 4 hr. at 100°, and the residues obtained by evaporation of the solutions were examined for amino acids. Portions of the products obtained after the acidification of the conjugates were similarly hydrolysed, and the products obtained examined in the same way. The results are shown in Table 1.

Examination of the amino acid conjugates obtained from 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene. Small portions of the conjugates were heated to 100° with 2N-HCl (0·5 ml.) for 5 min. The solutions were cooled and 10N-NaOH (1 ml.), ethanol (0·5 ml.) and ether (0·5 ml.) were added. Blue colours developing in the ether layer on shaking the mixture were specific for the presence of 2-tetralone (Straus & Rohrbacher, 1921).

Other portions of the conjugates were treated with Raney nickel and the products examined for naphthols and amino acids as described above. The products were also hydrolysed with HBr as before and the products examined for amino acids. The results are summarized in Table 2.

Other animal experiments. A rat with a biliary fistula was given *S*-(1-naphthyl)glutathione (100 mg.), suspended

in water (1 ml.), by intraperitoneal injection. The bile was collected for 48 hr. after the injection and examined for amino acid conjugates with the methods described above.

Two rats were each given a solution of *S*-(1:2:3:4-tetrahydro-2-hydroxy-1-naphthyl)glutathione (100 mg.) in water (1 ml.) daily for three successive days by intraperitoneal injection. The urines were collected, pooled and treated with charcoal as described by Boyland & Sims (1960). The material subsequently eluted from the charcoal with methanol containing 5% (v/v) of aq. NH_3 soln. (sp.gr. 0·88) was examined on paper chromatograms.

RESULTS

Biliary metabolites of naphthalene. The paper chromatograms of the bile of rats dosed with naphthalene showed a pattern of metabolites differing in a number of features from that seen on

Table 1. *Degradation products of the amino acid conjugates obtained from naphthalene*

Probable identity of the conjugate	Products obtained: With cold HCl*	By hydrolysis with HBr at 100°	By hydrogenolysis with Raney nickel and hydrolysis of the products with HBr†
N-Acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine	1-Naphthylmercapturic acid‡ *NN*-Diacetylcystine	*S*-(1-Naphthyl)-L-cysteine	Alanine
S-(1:2-Dihydro-2-hydroxy-1-naphthyl)-L-cysteine	*S*-(1-Naphthyl)-L-cysteine‡ Cystine	*S*-(1-Naphthyl)-L-cysteine	Alanine
S-(1:2-Dihydro-2-hydroxy-1-naphthyl)-L-cysteinylglycine	*S*-(1-Naphthyl)-L-cysteinyl-glycine‡ Substance, possibly oxidized cysteinylglycine	*S*-(1-Naphthyl)-L-cysteine Glycine	Alanine Glycine
S-(1:2-Dihydro-2-hydroxy-1-naphthyl)glutathione	*S*-(1-Naphthyl)glutathione Oxidized glutathione	*S*-(1-Naphthyl)-L-cysteine Glycine Glutamic acid	Alanine Glycine Glutamic acid
Two unidentified conjugates	Unidentified ninhydrin positive substances‡	*S*-(1-Naphthyl)-L-cysteine Glycine Glutamic acid Taurine	Alanine Glycine Glutamic acid Taurine

* All the conjugates gave rise to 1- and 2-naphthol.
† All the conjugates gave rise to 2- (but not 1-) naphthol.
‡ These products gave rise to the same amino acids on hydrolysis with HBr as the parent conjugates.

Table 2. *Chromatographic properties and degradation products of the amino acid conjugates derived from* 1:2-*dihydronaphthalene and* 1:2-*epoxy*-1:2:3:4-*tetrahydronaphthalene*

All the conjugates gave positive reactions with the $K_2Cr_2O_7$–$AgNO_3$ reagent.

Probable identity of the conjugate	R_F: Solvent 1	R_F: Solvent 2	Colour with ninhydrin	Products obtained: By hydrolysis with HBr at 100°*	By hydrogenolysis with Raney nickel and hydrolysis of the products with HBr at 100°
N-Acetyl-*S*-(1:2:3:4-tetrahydro-2-hydroxy-1-naphthyl)-L-cysteine	0·56	0·88	—	Cysteine†	Alanine
Unidentified metabolite	0·39	0·79	—	Cysteine† Glycine	Alanine Glycine
S-(1:2:3:4-Tetrahydro-2-hydroxy-1-naphthyl)-L-cysteine	0·57	0·72	Purple	Cysteine†	Alanine
S-(1:2:3:4-Tetrahydro-2-hydroxy-1-naphthyl)-L-cysteinylglycine	0·37	0·72	Brown, turning purple	Cysteine† Glycine	Alanine Glycine
S-(1:2:3:4-Tetrahydro-2-hydroxy-1-naphthyl)glutathione	0·25	0·45	Purple	Cysteine† Glycine Glutamic acid	Alanine Glycine Glutamic acid

* All metabolites gave a positive test (Straus & Rohrbacher, 1921) for 2-tetralone on heating with HBr or HCl for 5 min. at 100°.
† Cystine was often detected in the hydrolysates. It is presumed to be an artifact.

chromatograms of the urine of similarly treated animals. The bile did not contain either 1-naphthyl sulphate or the isomeric sulphuric esters of 1:2-dihydroxynaphthalene, although rats dosed with naphthalene excrete large amounts of these esters in the urine (Sims, 1959). 1-Naphthylglucosiduronic acid, the 1- and 2-glucosiduronic acids of *trans*-1:2-dihydro-1:2-dihydroxynaphthalene and substances believed to be the 1- and 2-glucosiduronic acids of 1:2-dihydroxynaphthalene were

Table 3. *Properties of the biliary naphthalene metabolites (other than the amino acid conjugates) on paper chromatograms*

Metabolite	R_F Solvent 1	R_F Solvent 3	Fluorescence	Colour with diazotized *p*-nitroaniline and Na_2CO_3: Immediate	Colour with diazotized *p*-nitroaniline and Na_2CO_3: After heating the chromatogram with 2N-HCl at 100° for 10 min.
1-Naphthol	0·97	0·62	Blue	Blue	Blue
2-Naphthol	0·97	0·55	Violet	Orange	Orange
trans-1:2-Dihydro-1:2-dihydroxy-naphthalene*	0·89	—	Dark-absorption	—	Blue
1-Naphthylglucosiduronic acid	0·28	—	Dark violet	—	Blue
trans-1:2-Dihydro-2-hydroxy-1-naphthylglucosiduronic acid*	0·18	—	Dark-absorption	Pale orange†	Blue
trans-1:2-Dihydro-1-hydroxy-2-naphthylglucosiduronic acid*	0·18	—	Dark-absorption	Blue†	Blue
2-Hydroxy-1-naphthylglucosiduronic acid	0·15	—	Bright blue	Yellow	Purple
1-Hydroxy-2-naphthylglucosiduronic acid	0·14	—	Bright blue	Blue	Purple

* The optical isomers of the compounds could not be separated on paper chromatograms.

† These colours are evidently due to decomposition of the metabolites by the reagent to give 1- or 2-naphthol. On two-dimensional chromatograms which were sprayed with 2N-HCl and allowed to dry between the first and second development (with solvent 1), compounds indistinguishable from 1- or 2-naphthol were detected.

Table 4. *Properties on paper chromatograms of the amino acid conjugates derived from naphthalene and their related acid-decomposition products*

All the compounds gave positive reactions with the $K_2Cr_2O_7$–$AgNO_3$ reagent.

Compounds	R_F Solvent 1	R_F Solvent 2	Fluorescence	Colour: With diazotized *p*-nitroaniline and Na_2CO_3	Colour: With ninhydrin
N-Acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine	0·37	0·84	Dark-absorption	Blue	—
1-Naphthylmercapturic acid	0·51	0·86	Pink*	—	—
S-(1:2-Dihydro-2-hydroxy-1-naphthyl)-L-cysteine	0·31	0·63	Dark-absorption	Blue	Purple
S-(1-Naphthyl)-L-cysteine	0·46	0·75	Pink*	—	Purple
S-(1:2-Dihydro-2-hydroxy-1-naphthyl-L-cysteinylglycine	0·25	0·62	Dark-absorption	Blue	Brown, then purple
S-(1-Naphthyl)-L-cysteinylglycine	0·40	0·74	Pink*	—	Brown, then purple
S-(1:2-Dihydro-2-hydroxy-1-naphthyl)-glutathione	0·10	0·47	Dark-absorption	Blue	Purple
S-(1-Naphthyl)glutathione	0·21	0·67	Pink*	—	Purple
Unidentified metabolites, possibly taurine conjugates 1	0·05	0·40	Dark-absorption	Blue	Purple
Unidentified metabolites, possibly taurine conjugates 2	0·01	0·35	Dark-absorption	Blue	Purple
Acid decomposition products of unidentified metabolites 1	0·10	0·49	Pink*	—	Purple
Acid decomposition products of unidentified metabolites 2	0·05	0·42	Pink*	—	Purple

* After exposure to NH_3 vapour.

readily identified by methods previously described (Boyland & Sims, 1957, 1958; Sims, 1959) (see Table 3). Small amounts of free 1- and 2-naphthol were always detected, but the amounts of free *trans*-1:2-dihydro-1:2-dihydroxynaphthalene found in the bile varied. Often none could be detected. A similar variation in the amounts of this diol in the urines of animals treated with naphthalene has been noted (Sims, 1959).

The main difference between the bile and urine of rats treated with naphthalene, however, was in the number of amino acid conjugates present. Only one such conjugate, the mercapturic acid *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine, has been found in urine (Boyland & Sims, 1958), whereas six were found in bile. The properties and degradation products of these conjugates are listed in Tables 4 and 1 and they are clearly related to each other, differing only in the nature of the amino acid side chain. Of the six, one was indistinguishable from the above mercapturic acid, one was indistinguishable from *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione, which is formed by the action of rat-liver slices on naphthalene (Booth *et al* 1960*b*), and a third was indistinguishable from *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine, which is formed from the above glutathione conjugate by rat-kidney homogenate (Booth *et al.* 1960 *b*). A fourth conjugate, which ran on chromatograms in a position intermediate to the glutathione and cysteine derivatives and which gave on acidification a product indistinguishable from *S*-(1-naphthyl)-L-cysteinylglycine, is believed to be *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteinylglycine. The hydrogenolysis and acid-hydrolysis products listed in Table 1 provide supporting evidence for these structures, whereas the acid-decomposition products were similar in every respect on paper chromatograms to the synthetic materials.

On one occasion, the fraction of bile (from a rat which had received a total of 750 mg. of naphthalene) containing the glutathione derivative was dissolved in water (3 ml.), and 1 ml. of the resulting solution acidified with HCl. After 24 hr. the solid was collected to yield *S*-(1-naphthyl)glutathione (15 mg.), m.p. and mixed m.p. 222–225° (decomp.), indistinguishable from the synthetic material on paper chromatograms. The infrared spectra of the two samples were identical, and both yielded compounds indistinguishable from glycine, glutamic acid and *S*-(1-naphthyl)cysteine on paper chromatograms after being heated to 100° with HBr (sp.gr. 1·7) for 4 hr.

The relative amounts of the four conjugates described above as determined by the sizes and intensities of their spots on paper chromatograms were somewhat variable, but usually the glutathione derivative was the predominant metabolite, whereas the mercapturic acid was often difficult to detect, particularly in the bile of animals which had received only one dose of naphthalene. A similar pattern of amino acid conjugates was observed in the bile of the animal treated with *S*-(1-naphthyl)-glutathione: much unchanged glutathione conjugate was found, together with smaller amounts of *S*-(1-naphthyl)-L-cysteinylglycine and *S*-(1-naphthyl)-L-cysteine and traces of 1-naphthylmercapturic acid.

The two remaining amino acid conjugates from the bile of animals treated with naphthalene were slower running than the glutathione conjugate on paper chromatograms, so presumably they have longer amino acid side chains than this metabolite. Apart from *S*-(1-naphthyl)cysteine, glutamic acid and glycine, the only amino acid found to be present in the hydrolysates of both of these conjugates was taurine, so that they may be taurine conjugates of *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione. Since only small amounts of these conjugates were present in bile, as compared with the other conjugates their structures must remain uncertain.

Biliary metabolites of 1:2-*dihydronaphthalene.* The bile from rats treated with this hydrocarbon contained small amounts of all the metabolites of naphthalene described above. This is not surprising since naphthalene metabolites have been detected in the urine of rabbits dosed with 1:2-dihydronaphthalene by Boyland & Sims (1960). Like this urine, the bile contained relatively large amounts of 2-naphthol, which may not have arisen by the same route as the naphthalene metabolites, since it is also a product of the action of rat-liver slices on 1:2-dihydronaphthalene (Booth *et al.* 1960*a*). The bile resembled that of animals treated with naphthalene in that no sulphuric esters were detected. *trans*-1:2:3:4-Tetrahydro-1:2-dihydroxynaphthalene and the corresponding glucosiduronic acid, which was isolated from the urine of treated rabbits by Boyland & Sims (1960), were detected in the bile.

As in the bile of rats treated with naphthalene, the most prominent feature of the biles of rats treated with 1:2-dihydronaphthalene was the number of compounds giving positive reactions with the $K_2Cr_2O_7$–$AgNO_3$ reagent. The properties and degradation products of these compounds are listed in Table 2. Of the five compounds detected, four were identified as the mercapturic acid, *N*-acetyl-*S*-(1:2:3:4-tetrahydro-2-hydroxy-1-naphthyl)-L-cysteine, and the corresponding cysteine, cysteinylglycine and glutathione derivatives. Of these, the mercapturic acid and the cysteine derivative have been found in the urine of animals treated with 1:2-dihydronaphthalene (Boyland & Sims, 1960), and the glutathione derivative is a

product of the action of rat-liver slices on 1:2-dihydronaphthalene (Booth *et al.* 1960*a*). On paper chromatograms the conjugates were indistinguishable from the corresponding synthetic compounds.

The fifth biliary metabolite was indistinguishable on paper chromatograms from a product previously detected in the urine of animals treated with 1:2-dihydronaphthalene (Boyland & Sims, 1960), which did not react with ninhydrin, and which yielded 2-tetralone, cysteine (and cystine) and glycine on acid-hydrolysis. A possible structure for this compound would be *N*-acetyl-*S*-(1:2:3:4-tetrahydro-2-hydroxy-1-naphthyl)-L-cysteinylglycine, but the product from the acetylation of the corresponding cysteinylglycine conjugate with acetic anhydride in aqueous sodium hydroxide (which also yielded 2-tetralone, cysteine and glycine on acid-hydrolysis) was not identical with the metabolite on paper chromatograms. The metabolite was not identical on paper chromatograms with the corresponding diketopiperazine derivative described above. Further evidence that the metabolite was closely related to the above amino acid derivatives was obtained by an examination on paper chromatograms of the urine of rats treated with *S*-(1:2:3:4-tetrahydro-2-hydroxy-1-naphthyl)-glutathione. The urine contained compounds indistinguishable from the above mercapturic acid, the cysteine and cysteinylglycine derivatives and the unidentified metabolite. A third possible structure of this metabolite would be the *N*-acetylglycylcysteine derivative, which would also yield 2-tetralone, cysteine and glycine on acid-hydrolysis. This could arise by ring-opening of the diketopiperazine derivative, followed by acetylation of the amino group.

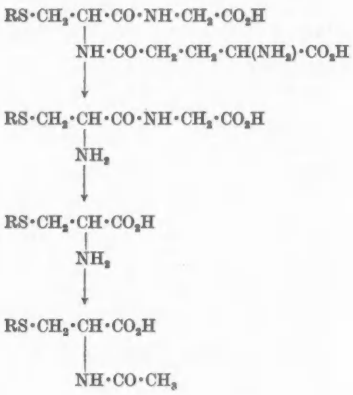
Biliary metabolites of 1:2-*epoxy*-1:2:3:4-*tetrahydronaphthalene.* These closely resembled those of 1:2-dihydronaphthalene except that the naphthalene metabolites mentioned above and 2-naphthol were not detected. The bile contained *trans*-1:2:3:4-tetrahydro-1:2-dihydroxynaphthalene and its glucosiduronic acid and compounds indistinguishable on paper chromatograms from the amino acid conjugates obtained from 1:2-dihydronaphthalene described above. The amounts of the various amino acid conjugates from both 1:2-dihydronaphthalene and the corresponding epoxide were variable, but usually the glutathione derivative was most prominent, whereas the mercapturic acid and the unidentified metabolite sometimes occurred only in traces, particularly in animals which had received few doses of the compounds. Substances corresponding to the supposed taurine conjugates derived from naphthalene were not detected in the bile of animals receiving 1:2-dihydronaphthalene or its epoxide.

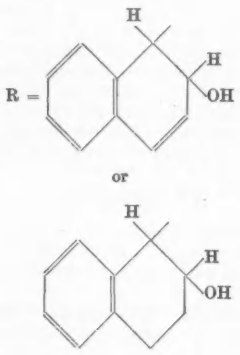
DISCUSSION

The presence of metabolites other than amino acid conjugates in the biles of rats treated with naphthalene, 1:2-dihydronaphthalene or 1:2-epoxy-1:2:3:4-tetrahydronaphthalene calls for little comment, since these metabolites are presumably formed in the liver and are known to be excreted in the urine. The absence of sulphuric esters in the biles of animals treated with the two hydrocarbons is remarkable since large amounts of these esters are excreted in the urines. Moreover, taurocholic acid, a sulphonic acid, is a normal constituent of bile and sulphuric esters of bile alcohols are present in the biles of lower vertebrates (cf. Haslewood, 1955).

The proposed structures for the amino acid conjugates found in bile after the administration of naphthalene, 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene are confirmed by the nature of their degradation products and they are clearly related to the mercapturic acids detected in the urine of animals after similar treatment. Moreover, the present work provides a connecting link between these metabolites and the glutathione conjugates formed from the same compounds in experiments *in vitro* with rat-liver slices (Booth *et al.* 1960*a*, *b*). In these experiments, the breakdown of the glutathione derivative to the cysteine derivative by rat-liver slices was not observed, but it could readily be brought about by rat-kidney homogenates. On the other hand, Bray, Franklin & James (1959) found that *S*-(*p*-chlorobenzyl)glutathione was converted into the corresponding *S*-cysteine derivative by guinea-pig-liver slices and by homogenates of guinea-pig, rat and rabbit liver. The intermediate formation of cysteinylglycine derivatives has not been observed in experiments *in vitro*. The absence of γ-glutamylcysteine derivatives in the biles of treated animals suggests that the breakdown of the glutathione conjugates proceeds in the same way as the breakdown of the glutathione itself, the first step being the loss of the γ-glutamyl groups, either by hydrolysis, by transfer to acceptors, or by cyclization to pyrrolidonecarboxylic acid (cf. Jocelyn, 1959). This is followed by the loss of glycine and the acetylation of the amino groups to give the mercapturic acids. The isolation of *S*-(1-naphthyl)-glutathione (liberated from its precursor by acid) provides direct evidence for the formation of a conjugate of this tripeptide in the body.

There is an interesting difference between the metabolism of naphthalene and that of 1:2-dihydronaphthalene and its related epoxide in that with naphthalene only the mercapturic acid, *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine, has been detected in the urine of treated





animals, whereas with the latter compounds both the corresponding cysteine conjugate and the mercapturic acid are always present. Moreover, the corresponding cysteinylglycine conjugate was detected in the urine of animals treated with *S*-(1:2:3:4-tetrahydro-2-hydroxy-1-naphthyl)glutathione. The unidentified metabolite described above was present in these urines as well as in the urines of animals treated with 1:2-dihydronaphthalene and its epoxide, but no analogous compound has been detected in the urines (or in the bile) of animals treated with naphthalene.

The amino acid conjugates obtained from naphthalene are all related to *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine, which breaks down with cold acid to give mainly 1-naphthylmercapturic acid (Boyland & Sims, 1958) together with small amounts of 1- and 2-naphthol and *NN*-diacetylcystine. Table 1 shows that in the breakdown of the related amino acid conjugates, side reactions yielded small amounts of the naphthols and oxidized forms of the sulphur-containing amino acids or peptides: the reduced forms (which would be expected) were not detected. Solutions of the conjugates did not give positive reactions for SH groups with sodium nitroprusside on acidification but always produced strong odours of naphthalene (although the hydrocarbon has not been detected chemically).

The formation of mercapturic acids from both naphthalene and 1:2-dihydronaphthalene appears to involve the reaction of intermediates, possibly epoxides (Booth *et al.* 1960*a*; Boyland & Sims, 1960) with glutathione. The glutathione conjugates are broken down to yield the cysteinylglycine and the cysteine derivatives and finally, after the acetylation of the amino groups, the mercapturic acids.

SUMMARY

1. The bile of rats treated with naphthalene contains 1- and 2-naphthol, 1-naphthylglucosiduronic acid, the 1- and 2-glucosiduronic acids of *trans*-1:2-dihydro-1:2-dihydroxynaphthalene and 1:2-dihydroxynaphthalene, and sometimes the free dihydrodihydroxy compound. Sulphuric esters were not detected.

2. The bile also contained *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione, together with the corresponding *S*-cysteinylglycine, *S*-cysteine and *N*-acetyl-*S*-cysteine conjugates. Two other conjugates present in small amounts appeared to be taurine conjugates of the glutathione derivative.

3. The bile of rats treated with 1:2-dihydronaphthalene contained all the above naphthalene metabolites, together with *S*-(1:2;3:4-tetrahydro-2-hydroxy-1-naphthyl)glutathione and the corresponding *S*-cysteinylglycine, *S*-cysteine and *N*-acetyl-*S*-cysteine conjugates. An unidentified metabolite, which yielded cysteine, glycine and 2-tetralone on acid-hydrolysis, appeared to be related to the above conjugates and it was also present in the urine of animals treated with the above glutathione conjugate. The bile also contained *trans*-1:2:3:4-tetrahydro-1:2-dihydroxynaphthalene and its glucosiduronic acid and relatively large amounts of 2-naphthol.

4. The bile of rats treated with 1:2-epoxy-1:2:3:4-tetrahydronaphthalene contained five amino acid conjugates indistinguishable from those present in the bile of rats treated with 1:2-dihydronaphthalene. *trans*-1:2:3:4-Tetrahydro-1:2-dihydroxynaphthalene and its glucosiduronic acid were also present.

5. A method for the establishment of bile fistulae in rats is described.

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Spectrophotometric Measurements on Ascorbic Acid and Their Use for the Estimation of Ascorbic Acid and Dehydroascorbic Acid in Plant Tissues

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The ultraviolet-light-absorption of ascorbic acid in neutral solutions was described by Herbert, Hirst, Percival, Reynolds & Smith (1933), Baird *et al.* (1934) and Morton (1942). Racker (1952) suggested application of spectrophotometry to enzymic microestimation of ascorbic acid or assay of ascorbic acid oxidases but did not investigate the conditions for such methods or the effects of pH on the absorption spectrum. Effects of pH were studied in detail by Daglish (1951), who described a spectrophotometric assay based on effects of pH on extinction. We have compared enzymic and chemical methods for estimating ascorbic acid and dehydroascorbic acid by spectrophotometric and visual-titration methods. Molar extinction coefficients of L-ascorbic acid found in this work were higher than those recorded by all previous workers except Lawendel (1956, 1957).

The spectrophotometric method we now describe was designed to estimate ascorbic acid in as little as 10 mg. of plant tissue. Between 1 and 75μg. of ascorbic acid or dehydroascorbic acid can be determined in 3 ml. of extract, 2μg. within about 5% and 10–20μg. within about 2% of the amount present. Non-enzymic methods of comparable sensitivity, with 2:6-dichlorophenolindophenol (Bessey, 1938; Glick, Alpert & Stecklein, 1953), are liable to interference, especially by reducing agents other than ascorbic acid. Chromatographic separation from reductones and thiols has been used by Chen, Isherwood & Mapson (1953) and by Strohecker, Heimann & Matt (1955). These methods also separate L-ascorbic acid from D-arabo- and D-gluco-ascorbic acid, but are lengthy and result in substantial losses with some plant tissues.

The estimation of dehydroascorbic acid with an indophenol dye by the difference in ascorbic acid found before and after reduction with hydrogen sulphide (Bessey, 1938) or with homocysteine (Hughes, 1954, 1956) is dependent on no other reducing compounds being produced by these treatments. We have compared the effects of reduction by hydrogen sulphide or homocysteine on the determination of dehydroascorbic acid by independent enzymic or chemical means.

EXPERIMENTAL

Materials

L-Ascorbic acid (British Drug Houses Ltd.) was found to be 99·4% pure by titration with KIO_3 in 4N-HCl in the presence of $CHCl_3$ (1 drop) to detect free I_2 at the end point

(Andrews, 1903). The melting point was 188° (uncorr.) with decomposition.

Solutions of dehydroascorbic acid were prepared as required by oxidation of ascorbic acid with bromine water in 2% (w/v) HPO_3 as described by the Association of Vitamin Chemists (1947). The solution was aerated to remove excess of Br_2. Measurements of light-absorption at 265 mμ before and after oxidation, and on subsequent reduction by H_2S at pH 3·0–3·5 (Levenson, Rosen & Hitchings, 1951) or by homocysteine at pH 6 (Hughes, 1956) were compared with titration by 2:6-dichlorophenol-indophenol reagent; they showed that both oxidation and the subsequent recovery of ascorbic acid by reduction were complete. A sample of solid dehydroascorbic acid (Sigma Chemical Co.) was found to be 80% pure by these tests.

Dihydroxyfumaric acid (Hartree, 1953), called dihydroxymaleic acid in earlier literature, was prepared by peroxidation of tartaric acid in the presence of ferrous sulphate (Powers, Tabakoglu & Sable, 1955). The product was recrystallized from aqueous methanol and dried *in vacuo*, m.p. 155° (uncorr.) in agreement with Fenton (1894); light-absorption maximum was 290 mμ (Hartree, 1953; Baraud, 1954). The molar extinction coefficient (ε) at 290 mμ immediately after dissolution in water was $7{\cdot}8 \times 10^3$, which is about 15% lower than the value (9×10^3) given by Hartree (1953).

Metaphosphoric acid solutions were freshly prepared by dilution of a 20% (w/v) solution. Copper was removed by extraction with 0·05% (w/v) diphenylthiocarbazone (dithizone) in CCl_4 (Parks, Hood, Hurwitz & Ellis, 1943). The effectiveness of this method was tested by adding known amounts of Cu^{2+} ions to a copper-free sample of HPO_3. Trisodium phosphate was free from copper or other metals that react with dithizone. Solutions of 2:6-dichlorophenolindophenol were standardized by rapid titration against pure ascorbic acid in 2% (w/v) HPO_3 (Harris & Olliver, 1942). Water was obtained by treatment of rain water through several ion-exchange resins including Bio-Deminrolit (Hewitt, 1952). It contained less than 0·0005 p.p.m. of Cu^{2+} ions. Hydrochloric acid (A.R. grade) was redistilled as a 6N-solution from Pyrex glass before use.

Cucumber ascorbic acid oxidase was obtained from the cell sap of cucumber fruits thawed after storing indefinitely in the frozen state at −18°. The fraction precipitated with ammonium sulphate between 65 and 75% saturation was used. This was stable for several days in solution at 0°. Addition of the enzyme to solutions of dihydroxyfumaric acid had no effect on the slow decrease in extinction of the latter at 290 mμ (about 4%/hr. for an aqueous 20 μM-solution). Dihydroxyfumaric acid-oxidase activity was therefore apparently absent. No dehydroascorbic acid-reductase activity was detected. The enzyme solutions usually contained 2 mg. of protein/ml. and the addition of 0·05 ml. of this solution to 3 ml. achieved the oxidation of 90 μg. of ascorbic acid present at a concentration of 30 μg./ml. Preparations should have an activity of at least 4 Snow & Zilva units (Snow & Zilva, 1938), corresponding to an initial change in extinction at 265 mμ of 0·2/min. in 3 ml. of solution containing 15–60 μg. of ascorbic acid.

Estimations of pH were made with a glass electrode and direct-reading pH meter (Electronic Instruments Ltd.). Ultraviolet-absorption measurements were made with a Hilger Uvispek spectrophotometer in 1 cm. silica cells and all readings were corrected for blank absorption.

Spectrophotometric determination of ascorbic acid and dehydroascorbic acid

Standard solutions were prepared by dissolving 40–100 mg. of ascorbic acid in 100 ml. of 2% (w/v) dithizone-extracted metaphosphoric acid. Suitable portions were taken as soon as possible, the pH was adjusted to 6·8 with a predetermined amount of trisodium phosphate and the volume made up to a convenient amount before measurement of absorption at 265 mμ. Fig. 1 shows the agreement between extinction values for ascorbic acid and for corresponding amounts of dehydroascorbic acid (prepared as described above) after subsequent reduction with homocysteine (as described below). The recovery after oxidation and subsequent reduction is not affected by using unpurified metaphosphoric acid.

Plant samples (0·05–1 g.) were ground immediately in 10–30 times their weight of 2% (w/v) fresh copper-free metaphosphoric acid and a small amount of Pyrex-glass powder in a glass homogenizer (Townson and Mercer Ltd.) attached to a stirrer motor controlled with a rheostat. The extract and washings were transferred to 5, 10 or 25 ml. flasks and 0·21 M-trisodium phosphate solution was added (0·66 vol. relative to the volume of HPO_3 used). The volume was made up with 2% (w/v) metaphosphoric acid–0·21 M-trisodium phosphate (3:2, v/v). The pH should be 7·3–7·4.

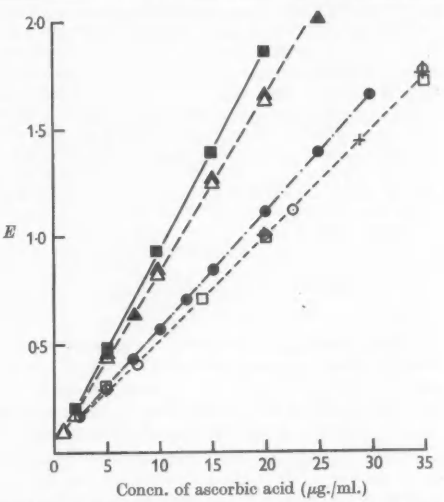

Fig. 1. Relationships between light-extinction (1 cm. depth) and concentrations of ascorbic acid (μg./ml.) in solutions of different composition: ■, at 265 mμ in purified 2% (w/v) metaphosphoric acid, neutralized to pH 6·8; ▲, at 265 mμ in unpurified metaphosphoric acid, neutralized to pH 6·8; △, as ▲ after oxidation with bromine water and subsequent reduction with homocysteine; ●, at 245 mμ in 2% (w/v) unpurified metaphosphoric acid, pH < 2; □, at 250 mμ in 2% (w/v) purified metaphosphoric acid, neutralized to pH 6·8; ○, at 250 mμ in 2% (w/v) metaphosphoric acid, pH < 2; +, at 250 mμ in water.

The extract was centrifuged for 3 min. at 3000*g* and the extinction (*A*) was measured at 265 mμ against a blank containing the metaphosphoric acid–trisodium phosphate mixture. Ascorbic acid-oxidase solution (0·05 ml.) was added to each cell and the extinction (*B*) was read after 15 min. To a similar portion was added 0·05 ml. of a solution containing 30 mg. of homocysteine/ml., which provides a molecular ratio of homocysteine to dehydroascorbic acid of between 50:1 and 250:1. The extinction (*C*) was read after 15 min. against a blank containing homocysteine and the metaphosphoric acid–trisodium phosphate mixture. The concentration of ascorbic acid (μg./ml.) was calculated from 10·80 ($A - 1{\cdot}02B$), and that of dehydroascorbic acid from 10·65 ($1{\cdot}02C - A$). The factors are based on $\epsilon\, 1{\cdot}65 \times 10^4$ for ascorbic acid and $\epsilon\ 2{\cdot}3 \times 10^2$ for dehydroascorbic acid at 265 mμ, their molecular weights and the dilutions in the cells. The initial extinction (*A*) varies greatly with plant species.

For 10–50 mg. samples, hydrogen sulphide was used in place of homocysteine. The sample was ground in 1·8 ml. of 2% (w/v) metaphosphoric acid and the extract transferred to a centrifuge tube. The homogenizer was washed out with 1·2 ml. of 0·21 M-trisodium phosphate solution and the extract and washings were centrifuged. The supernatant fluid was transferred as completely as possible to a 1 cm. absorption cell and reading *A* was made. Purified metaphosphoric acid (0·2 ml. of 40%, w/v) was added to lower the pH to 3·0–3·5 and hydrogen sulphide passed for 15 min., followed by N_2 for 45 min. Solid trisodium phosphate ($Na_3PO_4,12H_2O$) (copper-free) was added and dissolved with the aid of a fine glass rod to raise the pH to 7·0–7·4, and reading *C* was made. Enzyme solution (0·05 ml.) was stirred in and reading *B* was made after 15 min. Calculations are similar to those for the larger samples, but volume changes involved during transfer of the extract and addition of metaphosphoric acid, sodium phosphate and enzyme require the use of the factor 1·10 in place of 1·02.

RESULTS

Ultraviolet-light absorption of ascorbic acid solutions

Effects of pH. The absorption maximum of ascorbic acid was found at 265 mμ at pH > 6·8 and at 244 mμ at pH < 1·5, in agreement with Herbert *et al.* (1933), Baird *et al.* (1934), Morton (1942) and Daglish (1951). In unbuffered solutions the wavelength of the absorption maximum depends on concentration; it occurs at 264 mμ with 5μg./ml. (pH 4·70), at 256 mμ with 15μg./ml. (pH 4·35) and at 249 mμ with 35μg./ml. (pH 4·10), and the departure from Beer's law is considerable. In buffered solutions the wavelengths of the maxima at the pH values > 6·8 and < 1·5 are independent of the concentration of ascorbic acid. The light-extinction of ascorbic acid solutions in 2% (w/v) HPO_3, or in purified metaphosphate neutralized to pH 6·8, obeys Beer's law over the range 0–20μg./ml. (or over) as shown in Fig. 1. In unpurified, neutralized metaphosphoric acid, the deviation from Beer's law is negligible but the extinction may be low. The relationship between the values for $\lambda_{max.}$ and pH suggest a p*K* for ascorbic acid of about 4·25. This value is only slightly higher than that (4·15) found by Daglish (1951).

Isosbestic point. Three sets of absorption curves were obtained for different concentrations of ascorbic acid in 2% (w/v) HPO_3, in water and in metaphosphoric acid neutralized to pH 6·8. Their intersection at about 250 mμ confirmed the findings of Daglish (1951). An alternative and sensitive method for determination of the isosbestic point was provided by observation of the wavelength at which changes in pH had no effect on light-absorption values, since extinction at the isosbestic point is independent of the proportions of ionized and non-ionized forms determined by pH. The wavelength determined by this method was also 250 mμ, as shown in Fig. 2. Extinction at the isosbestic point obeyed Beer's law up to 35μg./ml. (or over) (Fig. 1), regardless of the wavelengths at which the absorption maxima occurred.

Molar extinction coefficients. Widely different values have been reported for the extinction of solutions of ascorbic acid (Lawendel, 1957). The values for ε found by us at first, from several determinations on different occasions, ranged between $1{\cdot}33 \times 10^4$ and $1{\cdot}55 \times 10^4$ with a mean of $1{\cdot}45 \times 10^4$ at 265 mμ and pH 6·8, and between $9{\cdot}95 \times 10^3$ and $1{\cdot}08 \times 10^4$ with a mean of $1{\cdot}03 \times 10^4$ at 245 mμ and pH 1–2. These values were greater than those commonly accepted (Baird *et al.* 1934; Morton, 1942; Brode, 1946) and similar to those ($1{\cdot}5 \times 10^4$, 265 mμ and $9{\cdot}6 \times 10^3$, 245 mμ) given by Daglish (1951).

While this work was in progress, Lawendel (1956, 1957) reported that when ascorbic acid is dissolved in media containing sorbitol at neutral

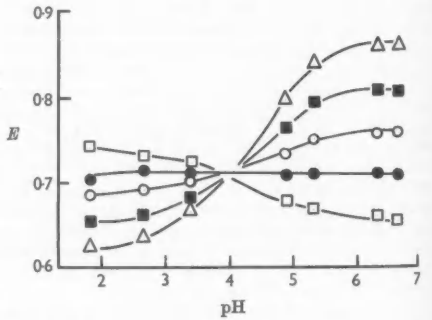

Fig. 2. Relationships between pH and light-extinction (1 cm. depth) of solutions of ascorbic acid (14 μg./ml.) at wavelengths near the isosbestic point: △, at 253 mμ; ■, at 252 mμ; ○, at 251 mμ; ●, at 250 mμ; □, at 249 mμ.

Table 1. *Extinction coefficients of ascorbic acid in aqueous solutions of different* pH *and composition*

pH	Medium	Wavelength (mμ)	$10^{-4}\,\epsilon$	$E^{1\%}_{1\,cm.}$
6·8 or above	Neutralized Purified HPO_3	265 (max.)	1·65	936
6·8	Neutralized Unpurified HPO_3	265 (max.)	1·45 Range: 1·35–1·55	823 766–880
1·5 or below	Purified HPO_3 or HCl	244 (max.)	1·05	595
< 2·0	Unpurified HPO_3	245	1·03 Range: 0·99–1·08	585 562–613
pH-independent	HPO_3, water only or neutralized HPO_3	250 (isosbestic point)	0·885	503

pH, higher extinction values than those previously recorded are obtained and the extinction in 'copper-free' media from which oxygen is excluded is similar to that observed in the presence of sorbitol. The values reported by Lawendel were $E^{1\%}_{1\,cm.}$ 916–940 at 265 mμ and pH 6·5, and 676–695 at 245 mμ in 0·1N-HCl. These correspond to ϵ $1{\cdot}612–1{\cdot}653 \times 10^4$ and $1{\cdot}190–1{\cdot}222 \times 10^4$ respectively.

As our results appeared low we sought to confirm Lawendel's observations with sorbitol, but without success. Tests with dithizone showed that variable trace amounts of copper were present in the metaphosphoric acid solutions and that the latter could be purified by dithizone extraction before adjustment of the pH to 6·8. By using dithizone-purified metaphosphoric acid values between $1{\cdot}63 \times 10^4$ and $1{\cdot}66 \times 10^4$ at 265 mμ were obtained under aerobic conditions at pH 6·8 without sorbitol. The calibration curve in Fig. 1 prepared by dissolving ascorbic acid in purified metaphosphoric acid before neutralization and dilution gave a value ϵ $1{\cdot}655 \times 10^4$ at 265 mμ and pH 6·8.

Repeated measurements between 243 and 245 mμ of light-absorption by solutions containing 5–35μg. of ascorbic acid/ml. in purified un-neutralized metaphosphoric acid or in either 0·1N- or N-HCl (copper-free) produced values for ϵ between $1{\cdot}01 \times 10^4$ and $1{\cdot}06 \times 10^4$, i.e. $E^{1\%}_{1\,cm.}$ was 573–602 and never approached values of 676–695 (Lawendel, 1957). Measurements with two spectrophotometers [Hilger Uvispek and Optika (UK)] indicated that the absorption maximum is close to 244 mμ, where the extinction is about 1·5% higher than at 245 mμ. Our measurements are recorded in Table 1. The extinction at 250 mμ is only about 4% greater than the value observed by Daglish (1951).

Estimation of ascorbic acid and dehydroascorbic acid

Comparisons between alternative procedures. Examples of results for small samples or quantities, for which H_2S was used, are given in Table 2. Results given in Table 3 show no consistent differences between homocysteine and H_2S as reducing agents or between the indophenol titration and the enzymic spectrophotometric methods. Use of bromine water to oxidize ascorbic acid in extracts caused errors due to changes in other substances absorbing light at 265 mμ. The treatment with H_2S sometimes resulted in turbidity, which caused errors unless the solution was filtered.

The control of pH is important in both homocysteine- and H_2S-reduction procedures. Levenson, Rosen & Hitchings (1951) found that reduction by H_2S is reversible, rapid and complete at pH 3·5, but that other irreversible reactions become significant below pH 3·0. The reduction with homocysteine is relatively slow at pH 6·5. We found that at pH 6·5 the reaction between homocysteine and dehydroascorbic acid in molar ratio 40:1 was only 90% complete in 30 min. at room temperature, whereas at pH 7·3 and above, with a molar ratio of only 10:1, the reaction was complete in less than 15 min. This is in agreement with observations at pH 7·2 by Hughes (1956). The pH values should not, however, exceed 7·5, as ascorbic acid is unstable when the pH is near 8.

Effect of dihydroxyfumaric acid. Dihydroxyfumaric acid interferes with the estimation of ascorbic acid by the indophenol method and, according to Snow & Zilva (1938), dihydroxyfumaric acid is oxidized by crude cucumber-oxidase extracts unless these are dialysed. As dihydroxyfumaric acid absorbs light at 265 mμ (Hartree, 1953; Baraud, 1954) it was necessary to test whether interference occurred. Cucumber oxidase fractionated with ammonium sulphate as described above produced no change in the slow rate of decrease in extinction at 265 or 290 mμ of solutions of dihydroxyfumaric acid prepared by us, and it is concluded from this that this compound does not interfere in the spectrophotometric estimation of ascorbic acid when the partially purified cucumber oxidase is used.

Recovery of ascorbic acid and dehydroascorbic acid. Addition of ascorbic acid or dehydroascorbic acid to metaphosphoric acid extracts show that satis-

factory recoveries were obtained (Table 4). When varying amounts of ascorbic acid were added to samples of fresh tissue immediately before grinding in metaphosphoric acid or when tissues were ground in metaphosphoric acid containing known concentrations of ascorbic acid there were losses of 20–35% of the amounts added. These losses were fully recovered as dehydroascorbic acid (Table 5). The ratio of ascorbic acid to dehydroascorbic acid in extracts of tissues to which no ascorbic acid had been added was higher than when ascorbic acid was added to tissues before grinding. Addition of ascorbic acid after grinding in metaphosphoric acid, or after centrifuging, resulted in recoveries of 94 and 96% respectively. Several alternative extraction methods were tried. These included the use of 10% (w/v) purified metaphosphoric acid, or of 2% or 10% (w/v) trichloroacetic acid saturated with ether, or plunging samples into boiling ethanol. None of these materially altered the proportions or amounts of ascorbic acid and dehydroascorbic acid subsequently found. The use of metaphosphoric acid solutions saturated with ethylenediaminetetra-acetic acid had no effect on the recovery of ascorbic acid added before grinding.

The method of estimation recently described by Barker & Mapson (1959), in which extraction is carried out under a stream of N_2 at $-3°$ in 4% (w/v) metaphosphoric acid, was compared with the one used here (Table 6). The differences in the ascorbic acid and dehydroascorbic acid contents found by the two methods were small.

DISCUSSION

The results obtained by us and by Lawendel (1956, 1957) show that the molar extinction coefficient of ascorbic acid at 265 mμ is much

Table 2. *Estimation of ascorbic acid and dehydroascorbic acid in separate samples of cauliflower leaf*

All extinction measurements were made on the same portion of solution for each sample and calculated from the formulae 10·80 ($A - 1{\cdot}1B$) for ascorbic acid and 10·65 ($1{\cdot}1C - A$) for dehydroascorbic acid.

		Ascorbic acid		Dehydroascorbic acid	
Wt. of tissue (mg.)	$E_{265\ m\mu}$	µg./ml. of extract	µg./g. of leaf	µg./ml. of extract	µg./g. of leaf
13·4 (in 3·0 ml.)	*A* 0·319	—	—	—	—
	B 0·098	2·29	513	—	—
	C 0·315	—	—	0·29	65
11·7 (in 3·0 ml.)	*A* 0·236	—	—	—	—
	B 0·074	1·68	431	—	—
	C 0·235	—	—	0·21	54
360 (in 100 ml. (i)*	*A* 0·418	—	—	—	—
	B 0·220	2·14	595	—	—
	C 0·425	—	—	0·53	147
(ii)	*A* 0·418	—	—	—	—
	B 0·224	2·12	589	—	—
	C 0·423	—	—	0·51	141

* (i) and (ii) are determinations on duplicate 3 ml. samples drawn from 100 ml. of extract of 360 mg. of the tissue corresponding to 10·8 mg. in 3·0 ml.

Table 3. *Estimation of ascorbic acid and dehydroascorbic acid by different procedures*

Values are given as µg./g. fresh wt. in separate experiments.

Fraction	Procedure	Cauliflower	Tomato
Ascorbic acid	Spectrophotometric	592 658 791 461 584	912
	Indophenol titration	603 648 778 474 598	909
Dehydroascorbic acid	Homocysteine; spectrophotometric	125 133 241 89 155	73
	H_2S; spectrophotometric	125 140 230 91 159	—
	Homocysteine; indophenol titration	125 138 225 95 176	—
	H_2S; indophenol titration	132 143 236 87 165	81
Total ascorbic acid + dehydroascorbic acid by addition*	Spectrophotometric (mean)	717 795 1030 552 741	985
	Indophenol titration (mean)	732 789 1010 565 769	990
Total ascorbic acid + dehydroascorbic acid by separate estimation†	H_2S; spectrophotometric	709 784 994 556 745	—

* The mean values for dehydroascorbic acid estimated by homocysteine or H_2S were used in the additions.

† Dehydroascorbic acid was reduced by H_2S before the total, as ascorbic acid, was estimated as described in the text for samples of 0·05–1 g.

Table 4. *Recovery of ascorbic acid or dehydroascorbic acid added to extracts of tomato- or cauliflower-leaf tissues*

	Added to extract (μg./ml.)	Found in extract (μg./ml.)	Expected (μg./ml.)	Recovery of added acid (%)
	Tomato leaf (40 mg./125 ml.)			
Ascorbic acid	Nil	0·80	—	—
	0·5	1·33	1·30	106
	1·0	1·72	1·80	92
	2·0	2·80	2·80	100
	4·0	4·74	4·80	99
	Tomato leaf (100 mg./125 ml.)			
Ascorbic acid	Nil	2·28	—	—
	6·0	8·10	8·28	97
	9·0	11·4	11·3	101
	Tomato leaf (100 mg./125 ml.)			
Dehydroascorbic acid	Nil	0·15	—	—
	1·0	1·13	1·15	98
	3·0	3·07	3·15	97
	5·0	5·01	5·15	97
	Tomato leaf (100 mg./42 ml.)			
Dehydroascorbic acid	Nil	0·53	—	—
	0·5	1·03	1·03	100
	1·0	1·56	1·53	102
	3·0	3·26	3·53	91
	5·0	5·26	5·53	95
	Cauliflower leaf (70 mg./83 ml.)			
Ascorbic acid	—	1·33	—	—
	2·48	3·85	3·81	102
	4·96	6·43	6·29	103
	9·92	11·1	11·3	99

Table 5. *Recovery of added ascorbic acid as ascorbic acid and dehydroascorbic acid*

Ascorbic acid was added to 250 mg. of cauliflower leaf before it was ground in 25 ml. of copper-free metaphosphoric acid.

Concn. in leaf extracts without added ascorbic acid (μg./ml.)			Found in leaf extracts with added ascorbic acid (μg./ml.)			Recovery of added ascorbic acid (%)	Ascorbic acid/dehydroascorbic acid in extracts	
Ascorbic acid	Dehydroascorbic acid	Ascorbic acid added (μg./ml.)	Ascorbic acid	Dehydroascorbic acid	Total		Without added ascorbic acid	With added ascorbic acid
7·15	1·54	5	10·4	3·20	13·6	65	4·64	3·26
6·70	1·12	10	14·5	3·10	17·6	78	6·01	4·70

Table 6. *Comparison of results of extraction by the method of Barker & Mapson* (1959) (1) *and the method described in the text* (2)

Ascorbic acid and dehydroascorbic acid were determined spectrophotometrically in different samples of cauliflower leaf weighing 250 mg.

Sample	I		II		III	
Method	1	2	1	2	1	2
Ascorbic acid (μg./g.)	775	763	663	640	643	631
Dehydroascorbic acid (μg./g.)	80	74	58	61	100	102
Ascorbic acid/dehydroascorbic acid	9·7	10·3	11·4	10·5	6·4	6·2

higher than that previously recorded. The maximum value recorded here is $1{\cdot}66 \times 10^4$. Since values tend to be low, due to oxidation, the value ϵ $1{\cdot}65 \times 10^4$ at 265 mμ and pH 6·8 or above, which agrees with that reported by Lawendel, is accordingly proposed. We are unable, however, to confirm Lawendel's figure for ϵ at 245 mμ in 0·1N-HCl or in metaphosphoric acid or under nitrogen in acid, and we propose the value ϵ $1{\cdot}05 \times 10^4$ at 244 mμ and pH 1·5 or below. This is close to values reported by several workers, who nevertheless recorded relatively low values for corresponding measurements at 265 mμ and pH > 6·5. In the work described here care was taken to exclude as far as possible traces of copper and other heavy metals from water and reagents. Glass-distilled water of probably low copper content was used by many who recorded low values for ϵ at 265 mμ in earlier work. However, the buffer reagents used to produce the pH values above 6·5 were often not purified, and heavy-metal-catalysed oxidation of ascorbic acid probably occurred. By using metaphosphoric acid and trisodium phosphate which were free of copper, catalysis of oxidation by Cu^{2+} ions was avoided. No changes in extinction were observed over several hours for plant extracts in 2% (w/v) purified metaphosphoric acid (pH 1·5). At pH 7 in neutralized purified metaphosphoric acid a slow decrease in extinction at 265 mμ was observed, amounting only to 1% in the first 15 min. and thereafter to about 0·5%/hr. over 24 hr. The error to be expected from these changes during the period of the determination is therefore negligible. It might be concluded that as copper and oxygen have greater effects on loss of ascorbic acid at pH values of about 6·5–7·0 than in acid solutions, light-extinction values would be less inaccurate at 245 than at 265 mμ at the appropriate pH values. We are also unable to confirm Lawendel's observations on the effect of sorbitol. It is possible that his sample contained a copper-chelating impurity which served to protect ascorbic acid from catalytic oxidation.

The analytical method described above has the advantage of greater specificity than methods based on indophenol and is at least as sensitive and as easy to apply. The change in extinction at 265 mμ (pH 7·3) due to oxidation with cucumber oxidase is about double that produced at 265 mμ by changing the pH from 3·0 to 4·6; the present method is thus proportionately more sensitive than that proposed by Daglish (1951), which was based on this effect of pH.

The extent of interference by reductones and reductic acid has not been established. The absorption maximum of reductone is at 285 mμ at pH 5·7, according to Euler, Hasselquist & Ceder (1953), who gave no data for extinction in relation to concentration. The results obtained by Snow & Zilva (1938) in a comparative study of the oxidation of ascorbic acid, reductone and reductic acid by ascorbic acid oxidase indicate the extent to which possible interference by reductone may occur and how it may be minimized. They found that the rates of oxidation of reductone and of reductic acid by the oxidase were much less than those of equivalent concentrations of ascorbic acid, and further that the differences in these rates become more marked as the concentrations of the respective substrates are decreased. Their data indicate that at concentrations of ascorbic acid of 10–20μg./ml. the rate for ascorbic acid should be at least 20 times that for equivalent concentrations of reductone or reductic acid. Enzyme concentration also affects rates of oxidation of the substrates in a differential manner: increasing enzyme concentration increases the rate of oxidation of ascorbic acid relative to that of equivalent concentrations of reductone. For this reason enzyme preparations of the activity specified in the Experimental section should be used and the reaction time kept to a minimum (usually 10–15 min.). Under these conditions, where ascorbic acid concentrations of the order of 0·1 mM or less are involved, the error caused by the presence of equivalent concentrations of reductone or reductic acid might be as low as 5%.

Mapson (1958) queried whether any existing method provided a valid estimate of the proportions of ascorbic acid and dehydroascorbic acid in intact cells. It would appear from Tables 4 and 5 that added ascorbic acid is converted into dehydroascorbic acid during extraction, and that this reaction occurs to a relatively greater extent than in unsupplemented control extracts. Whether cellular ascorbic acid is also converted into dehydroascorbic acid during grinding remains unsettled. In attempts to elucidate this point, Barker & Mapson (1959) investigated effects of different extraction procedures on the ratio ascorbic acid/dehydroascorbic acid, especially with reference to the apparent content of dehydroascorbic acid. The method finally evolved by them, to which reference was made at the end of the preceding section, was designed to minimize the production of dehydroascorbic acid as an artifact resulting from enzymic oxidation, by exclusion of air and extraction at a low temperature. The comparisons shown in Table 6, which were made at the suggestion of Dr L. W. Mapson, showed no consistent or notable differences in the results obtained by the two methods. Although we consider that atmospheric oxidation catalysed by ionic copper introduced by the extracting medium can be discounted as the cause of the production of dehydroascorbic acid, it is possible that copper compounds extracted from

the plant tissues may catalyse the oxidation of ascorbic acid, at the time of cell rupture. Attempts to exclude atmospheric oxygen by saturating the extracting medium with nitrogen and flushing with nitrogen during extraction in an open vessel (Barker & Mapson, 1959) may not entirely prevent the access of oxygen. The oxygen consumption involved in the production of the amounts of dehydroascorbic acid recorded in Table 6 would in fact amount to only about 3μl. for 250 mg. of leaf tissue if hydrogen peroxide were produced and only 1·5μl. if peroxide were not formed. The relative stability of ascorbic acid in purified metaphosphoric acid extracts in air and the similarity in the amounts of dehydroascorbic acid obtained by the two methods compared in Table 6 suggest, however, that if dehydroascorbic acid is produced as an artifact in plant extracts, through the activity of ascorbic oxidase or indirectly of phenol oxidase enzymes, the reaction occurs for a short period only, at the time of cell rupture. In practice this reaction will be minimized by avoiding mechanical injury of the tissues as far as possible before extraction. 'Pickling' for a short time before extraction in an acid reagent which chelates copper and also penetrates rapidly might be advantageous, but has not been tried.

SUMMARY

1. L-Ascorbic acid at pH 6·8 or over has $\epsilon_{max.}$ (265 mμ) of $1{\cdot}65$–$1{\cdot}655 \times 10^4$. This value agrees with that of Lawendel (1956) but an effect of sorbitol reported by him could not be confirmed.

2. At pH between 1·0 and 2·0, in metaphosphoric acid or in hydrochloric acid, $\epsilon_{max.}$ (244 mμ) is $1{\cdot}05 \times 10^4$. This value is about 15% lower than that reported by Lawendel.

3. An isosbestic point which is independent of pH occurs at 250 mμ where ϵ is $8{\cdot}85 \times 10^3$.

4. Light-extinctions obey Beer's law at 265 and 245 mμ for concentrations of ascorbic acid up to 35μg./ml. in buffered solutions of pH 6·8 or above and pH 1–2 respectively, and at 250 mμ regardless of these pH limits.

5. A spectrophotometric method for the estimation of small amounts of ascorbic acid and dehydroascorbic acid in extracts of leaf tissues is described.

6. Satisfactory agreement was obtained between (*a*) indophenol titrations and (*b*) spectrophotometric changes resulting from oxidation by cucumber oxidase, and subsequent reduction of dehydroascorbic acid by either homocysteine or hydrogen sulphide.

7. Good recoveries of ascorbic acid and dehydroascorbic acid were obtained when small amounts were added to extracts after grinding. When such additions were made before extraction, losses of ascorbic acid up to 35% occurred and these were recovered as dehydroascorbic acid. The conversion of ascorbic acid into dehydroascorbic acid during extraction is greater for added ascorbic acid present in an extracellular location than for that present within the cells.

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Studies on the Effect of Anterior-Pituitary Growth Hormone on Oxidative Phosphorylation in Rat-Liver Mitochondria

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Injection of anterior-pituitary growth hormone into the intact rat causes changes of metabolism leading to a greater oxidation of fat (Young, 1945; Greenbaum & McLean, 1953) and a sparing of protein (Young, 1945; Li, Simpson & Evans, 1948). The mechanism whereby these changes are effected is unknown. The diverse nature of the metabolic changes induced by the action of the hormone make it seem unlikely that they could all arise as the result of the modification of a single specific enzyme and it appeared more profitable to investigate the possibility that the effects are mediated by some primary change of a more general character.

The change could involve (i) an effect on the permeability of the cell wall itself [as has been suggested by Kipnis & Cori (1957) for the action of insulin] or on the membrane of the subcellular structures [as has been suggested by Tapley & Cooper (1956) for the action of thyroxin], or (ii) the action could be on an ubiquitous system such as oxidative phosphorylation which could modify energy relationships within the cell. Park, Meriwether & Park (1958) have suggested that thyroxine acts directly on the enzyme system carrying out oxidative phosphorylation.

In the experiments reported here it has been shown that growth hormone uncouples phosphorylation when D(−)-β-hydroxybutyrate was oxidized by rat-liver mitochondria. (Throughout this paper the lowered efficiency of oxidative phosphorylation is referred to as an 'uncoupling' for the sake of brevity, although it is realized that the effect described here is not that usually implied by the use of this term. The efficiency here seems to be lowered not by any reduction in the rate of phosphorylation but by the appearance of additional oxidation.) It has also been shown that growth hormone in very low concentration causes striking changes in mitochondrial permeability.

METHODS

Animals. Female hooded Norwegian rats were used when their body weight had reached a plateau value of 180–200 g., i.e. at about 10 weeks old.

Hormones. The growth-hormone preparation used in the bulk of these experiments was a twice-recrystallized hormone prepared from ox-pituitary glands by the method of Wilhelmi, Fishman & Russell (1948). In addition two commercial preparations [Armour Somar A, a gift from the Endocrine Study Section of the National Institutes of Health, U.S.A., prepared by the same method, and a preparation, Batch no. 5609:2, a gift from Ferring AB, Malmo, made by the method of Raben & Westermeyer (1951)] were also used in some of the experiments on mitochondrial permeability. Other hormones used were insulin, adrenocorticotrophic hormone and prolactin (all gifts from the Biological Standards Division of the Medical Research Council). For the experiments on oxidative phosphorylation the growth hormone was dissolved in 0·9% NaCl soln. (2 mg./ml.) and 1 mg. was injected intraperitoneally 4 hr. before the animal was killed.

Reagents. Adenosine di- and tri-phosphate (ADP, ATP), oxidized and reduced diphosphopyridine nucleotide (DPN, DPNH) and triphosphopyridine nucleotide (TPN) were commercial preparations from the Sigma Chemical Co., Mo., U.S.A. Hexokinase was prepared by the method of Berger, Slein, Colowick & Cori (1945) from a yeast autolysate prepared by the method of Bailey & Webb (1948). It was purified as far as stage 3 of the Berger *et al.* (1945) procedure. Glucose 6-phosphate dehydrogenase was prepared by the method of Kornberg (1950). The method for preparing the (+)- and (−)-isomers of β-hydroxybutyrate was that of Lehninger & Greville (1953), which is based on an unpublished method by Dr H. T. Clarke.

Preparation of mitochondria and of 'digitonin particles'. Liver mitochondria were prepared as described by Schneider (1948) in 0·25 M-sucrose containing 10 μM-ethylenediaminetetra-acetic acid (EDTA). The final mitochondrial pellet was washed once with 0·25 M-sucrose and then suspended in 0·25 M-sucrose to give an 8% suspension (i.e. the mitochondria from 1 g. of liver suspended in 12·5 ml. of sucrose). For experiments on mitochondrial permeability the mitochondrial suspension was prepared in 0·44 M-sucrose as described by Tapley (1956).

Digitonin particles were prepared essentially according to the method described by Devlin & Lehninger (1956). With a 1:10 homogenate of three rat livers, nuclei and cellular debris were removed (two centrifugings at 900 *g* for 10 min.) and the mitochondria were collected by centrifuging at 9000 *g* for 10 min. The mitochondrial pellet was twice washed and re-sedimented in 0·25 M-sucrose. The final mitochondrial pellet was suspended in 2·25 ml. of 0·05 M-sucrose, and 1·5 ml. of an ice-cold 2% solution of recrystallized digitonin in 0·05 M-sucrose was added over a period of 5 min. with continuous stirring. The suspension was allowed to stand at 0° for 20 min. and then diluted to 13 ml. with 0·25 M-sucrose. The suspension was then centrifuged at 40 000 *g* for 20 min. and the supernatant from this

operation collected and centrifuged again at 100 000 *g* for 30 min. The resulting pellet was resuspended in water (1 ml. ≡ 4 g. of liver) and homogenized. Particles prepared in this manner (suggested in a personal communication from Professor A. L. Lehninger) differed from those prepared according to Cooper & Lehninger (1956) in maintaining a constant rate of phosphorylation for only about 10 min. instead of 15 min. and in being unaffected by Mg^{2+} ions at a concentration of 15 mM, whereas this concentration of Mg^{2+} ions actively uncoupled oxidative phosphorylation in the Cooper & Lehninger (1956) preparation.

Measurement of oxidative phosphorylation. For the measurement of oxidative phosphorylation with β-hydroxybutyrate, the phosphate esterified was trapped as glucose 6-phosphate, which was subsequently estimated by measuring the TPNH formed during the oxidation of the glucose 6-phosphate by glucose 6-phosphate dehydrogenase.

The rate of oxidation was measured by the rate of formation of acetoacetate since this was the only product of the oxidation when L-, D- or DL-β-hydroxybutyrate was used (see below).

The incubation mixture contained: 0·3 ml. of 0·3 M-glucose; 0·1 ml. of 0·04 M-ADP (sodium salt); 0·1 ml. of 0·3 mM-cytochrome *c*; 0·5 ml. of 0·1 M-sodium phosphate buffer, pH 7·4; 0·05 ml. of hexokinase solution (50 units of hexokinase activity); 0·1 ml. of 0·2 M-$MgSO_4$; 0·05 ml. of 1·5 M-KCl; 0·1 ml. of 0·1 M-EDTA (sodium salt); 0·2 ml. of bovine-plasma albumin (containing 7·5 mg. of albumin/ml.) and 0·3 ml. of 0·1 M-D-, L- or DL-β-hydroxybutyric acid. The mixture was equilibrated at 27° for 5 min., after which 1 ml. of the 8% mitochondrial suspension, immediately followed by 0·2 ml. of 0·02 M-NaF, was added and the whole was incubated for 5 min. At the end of this time 2 ml. was removed and pipetted into 1 ml. of ice-cold 9% perchloric acid. Controls, containing no substrate, were similarly treated. The precipitated protein was removed and the excess of perchloric acid then precipitated as the potassium salt. The final supernatant was analysed for acetoacetate and glucose 6-phosphate.

Acetoacetate was measured by the method of Greenberg & Lester (1944). Glucose 6-phosphate was measured in the following system: 0·3 ml. of the sample containing glucose 6-phosphate; 0·6 ml. of 0·25 M-glycylglycine buffer, pH 7·6; 0·6 ml. of 0·1 M-$MgCl_2$; 1·1 ml. of water; 0·2 ml. of TPN solution containing 2 μmoles; 0·2 ml. of glucose 6-phosphate dehydrogenase (10 mg./ml. of 0·1% albumin in 0·01 M-glycylglycine). The preparation of glucose 6-phosphate dehydrogenase used was virtually free of 6-phosphogluconate-dehydrogenase activity. The TPN was added last and the change in extinction at 340 mμ was recorded 10 min. after the addition of the TPN.

Measurement of mitochondrial permeability. The effect of hormones and proteins on mitochondrial permeability was determined by adding 0·05 ml. of a 50% mitochondrial suspension in 0·44 M-sucrose to 2·95 ml. of a sucrose solution containing the protein or hormone in a cuvette. The concentration of sucrose in the cuvette was so adjusted that the final concentration of the 3 ml. of solution was 0·25 M. At values above 0·25 M-sucrose swelling is inhibited (Lehninger, 1959).

An initial spectrophotometric reading of about 0·5–0·6 at a wavelength of 520 mμ was obtained and the changes in extinction were recorded over 25 min. The cuvette always included 0·1 ml. of 1·5 M-tris buffer (pH 7·4), which effectively precluded any change of pH. In experiments involving comparison of the activity of various hormones in producing an increased permeability of the mitochondria, the activity was estimated from the change in extinction between the fifth and fifteenth minutes.

Measurement of the pyridine nucleotide content of isolated mitochondria. In experiments where the DPN and DPNH contents of the isolated mitochondria were measured, the mitochondria from a whole liver homogenized in 0·25 M-sucrose containing 0·03 M-nicotinamide were isolated as described above and the DPN and DPNH contents of the entire mitochondrial pellet were estimated as described by Ciotti & Kaplan (1957).

RESULTS

P/O *ratios in liver mitochondria isolated from growth-hormone-treated rats.* The effect of the injection of 1 mg. of growth hormone into rats 4 hr. before death on the P/O ratio of isolated mitochondria oxidizing DL-β-hydroxybutyric acid is shown in Table 1.

It is apparent that there is a considerable decrease in the P/O ratio as a result of the growth-hormone injection and that this fall can be entirely ascribed to the increase in oxygen uptake. The amount of phosphorylation is almost identical in control and treated animals. To test the effect of protein itself Armour bovine-plasma albumin was injected in amounts of up to 5 mg., but even at the highest dose used this had no effect on either oxygen uptake or the rate of phosphorylation (Table 1).

The one-step oxidation of L-β-hydroxybutyrate is equivalent to the production and oxidation of DPNH (Lehninger, 1946), and for this reason the P/O ratio of the oxidation of this latter substance by mitochondria from normal and growth-hormone-treated rats was also measured. Surprisingly, no effect was found of the growth hormone causing a lowering of the P/O ratio. The observed values were $1·77 \pm 0·02$ for controls and $1·79 \pm 0·04$ for treated rats (five rats in each group). When the P/O ratio of glutamate was measured under similar conditions, once again no effect of growth hormone on the ratio could be observed ($2·31 \pm 0·05$ for controls and $2·29 \pm 0·07$ for treated rats: six rats were used in each group).

Resolution of the DL-β-hydroxybutyrate into the D- and L-salts showed that, under the conditions used here, only the L-β-hydroxybutyrate is oxidized with the D-salt being completely inactive. Thus of the three oxidation systems used here only the L-β-hydroxybutyric acid dehydrogenase is affected by growth hormone.

P/O *ratios of digitonin-particles from liver mitochondria isolated from normal and growth-hormone-treated rats.* A summary of the results showing the effect of pretreatment of rats with growth hormone on the rate of oxidation of DL-β-hydroxybutyrate

Table 1. P/O *ratios in liver mitochondria from normal and growth-hormone-treated rats*

Substrate DL-β-hydroxybutyrate. System: 0·3 ml. of 0·3 M-glucose; 0·1 ml. of 0·04 M-ADP; 0·1 ml. of 0·3 mM-cytochrome *c*; 0·5 ml. of 0·1 M-sodium phosphate buffer, pH 7·4; 0·1 ml. of 0·2 M-$MgSO_4$; 0·05 ml. of 1·5 M-KCl; 0·1 ml. of 0·1 M-EDTA, pH 7·4; 0·3 ml. of 0·1 M-DL-β-hydroxybutyrate; 0·05 ml. of hexokinase solution (≡ 50 units of hexokinase activity); 0·2 ml. of bovine-plasma albumin (7·5 mg./ml.); 0·2 ml. of 0·02 M-NaF and 1 ml. of 8% suspension of liver mitochondria. Temperature, 27°. Time, 5 min. 1·0 mg. of growth hormone or 1·0 mg. of albumin was injected 4 hr. before killing.

Treatment	No. of animals	Acetoacetate (μmole) found (μatom of O utilized)	Glucose 6-phosphate found (μmole)	P/O ratio
Mitochondria				
Control	7	0·256 ± 0·02	0·663 ± 0·05	2·61 ± 0·07
Growth hormone	7	0·361 ± 0·02	0·700 ± 0·05	1·96 ± 0·06
Albumin	3	0·260 ± 0·02	0·675 ± 0·06	2·62 ± 0·07
P (hormone-treated v. controls)		0·006	Not significant	0·001
Digitonin particles				
Control	4*	0·374 ± 0·04	0·664 ± 0·05	1·78 ± 0·13
Growth hormone	4*	0·350 ± 0·04	0·616 ± 0·05	1·76 ± 0·12
P (hormone-treated v. controls)		Not significant	Not significant	Not significant

* Each sample consists of the combined livers of three rats.

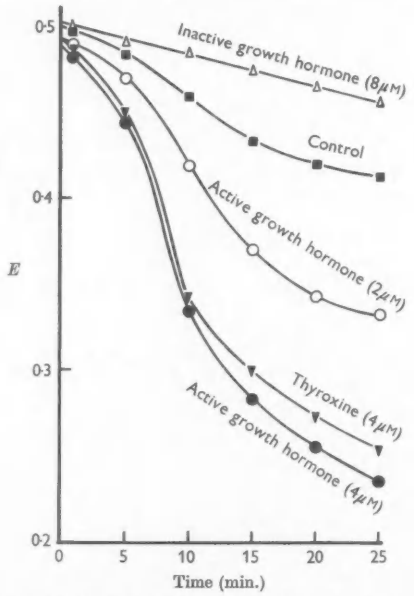

Fig. 1. Effect of two batches of growth hormone on the swelling of liver mitochondria suspended in 0·25 M-sucrose. △, 8 μM-Inactive Somar A growth hormone; ○, 2 μM-, and ●, 4 μM-, active growth hormone; ▼, 4 μM-thyroxine, inserted for comparison; ■, control curve: no additions.

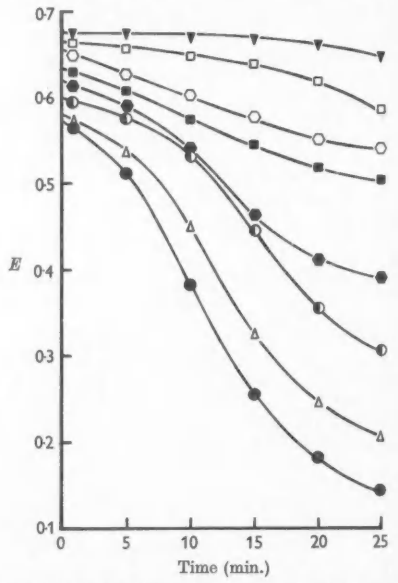

Fig. 2. Effect of the addition of 1 mg. of various proteins to 3 ml. of a suspension of liver mitochondria in 0·25 M-sucrose. ▼, Bovine-plasma albumin; □, casein; ○, prolactin; ●, adrenocorticotrophic hormone; ◐, insulin; ◉, growth hormone; △, 4 μM-thyroxine, inserted for comparison; ■, control curve: no additions.

by digitonin particles from rat-liver mitochondria is shown in Table 1.

It will be seen that, with DL-β-hydroxybutyrate as substrate, neither the oxygen uptake nor the rate of phosphorylation was different in the particles from the injected rats from that found in the particles of livers from control rats. It would appear that removal of the mitochondrial membrane abolished the effect of growth hormone on the efficiency of oxidative phosphorylation.

Growth hormone and mitochondrial permeability. The failure to observe an effect of growth hormone on the P/O ratio in digitonin particles drew attention to an apparent similarity between the action of growth hormone and of thyroxine in uncoupling oxidative phosphorylation, in that both hormones are active in this respect on the intact mitochondrion but had no effect on the digitonin particle. In view of the suggestion of Lehninger (1956) that the effect of thyroxine on oxidative phosphorylation was secondary to its action on mitochondrial permeability it became of considerable interest to examine whether growth hormone had an effect similar to that of thyroxine on the mitochondrial membrane.

Fig. 1 shows the effect of suspending mitochondria from a normal rat, isolated in 0·44 M-sucrose, in a medium of 0·25 M-sucrose containing growth hormone.

Growth hormone, at a concentration of 4μM, causes a profound decrease in extinction. Concentrations as low as 0·02μM have also been shown to produce a quite detectable effect. On a molar basis (assuming a molecular weight of 44 000 for growth hormone) some of the growth-hormone samples used have been about ten times as active as thyroxine.

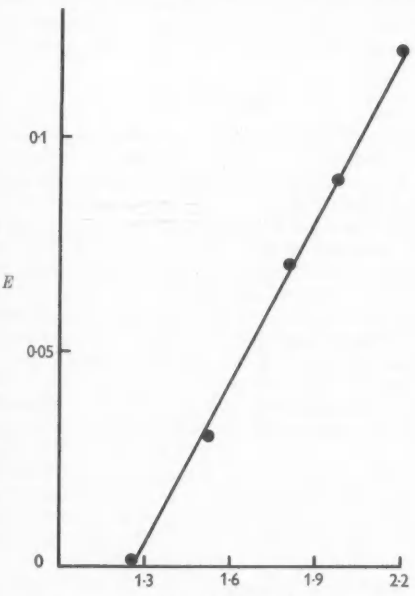

Fig. 3. log dose–response curve relating dose of growth hormone in the cuvette to change of extinction of a suspension of liver mitochondria in 0·25 M-sucrose over a period of 15 min. (Each point represents the mean of 36 determinations.)

The property of inducing swelling in isolated mitochondria appears to be closely related to the growth-promoting properties of the hormone preparation *in vivo*. When a number of batches of the hormone, of varying degrees of growth-promoting potency, were tested on isolated mitochondria, the property of causing swelling was shown only by those preparations which could cause growth of the female rat whose weight had reached a plateau value, and was only weakly present in those preparations which had a low activity in the living animal. One preparation which possessed virtually no growth-promoting activity (Armour, Somar A) actually stabilized the mitochondria either against spontaneous swelling (see Fig. 1) or against the swelling action of more active batches of growth hormone. In this respect the Armour preparation resembled the behaviour of a number of unspecific proteins such as bovine-plasma albumin, globulin, globin, lactoglobulin, casein, etc.

Of the many proteins tested, only two, insulin and adrenocorticotrophic hormone, induced swelling although both of these hormones were considerably less active than growth hormone itself (Fig. 2). If the change in extinction of the mitochondrial suspension produced by the addition of growth hormone is measured between the fifth and fifteenth minutes after mixing (corrected for spontaneous swelling) and plotted against log dose of the hormone, a typical dose–response curve is obtained (Fig. 3). Although this finding could provide the basis for a very sensitive assay of pure growth hormone it cannot, unfortunately, be used as an assay of the hormone in biological materials since the presence of small quantities of other protein (e.g. 2μM-plasma albumin) completely inhibits the promotion of swelling by growth hormone.

The resemblance between growth hormone and thyroxine both with respect to the effect on oxidative phosphorylation and on mitochondrial permeability prompted the speculation that the primary effect of growth hormone, like that of thyroxine (Lehninger, 1956), might be on the mitochondrial membrane. This point has been investigated (i) by observing whether there were changes in the permeability of mitochondria in which uncoupling had been demonstrated, and (ii) by testing whether uncoupling necessarily followed when an increased permeability was induced *in vitro*.

Mitochondrial permeability in vivo. Since mitochondria isolated from the liver of a rat treated 4 hr. previously with growth hormone exhibited a lowered P/O ratio (see Table 1) it might be expected, on the above-mentioned basis, that they

would show increased permeability. This point was examined by comparing the degree of swelling of mitochondria, isolated in 0·44 M-sucrose, from normal and growth-hormone-treated rats when they were exposed to (*a*) 0·25 M-sucrose, and (*b*) 0·25 M-sucrose containing various concentrations of growth hormone. Under both these sets of conditions the mitochondria isolated from the hormone-treated animals behaved in a manner identical with that of controls.

It might be anticipated also that if growth hormone increased mitochondrial permeability *in vivo* the mitochondria isolated from animals so treated would be larger than those obtained from normal rats. It is not possible to compare sizes of mitochondria by direct visual methods but a comparison is possible on calculation of the extinction/mg. of mitochondrial N. (This rests on the assumption that changes of factors such as asymmetry and surface structure play only a minor role in decreasing extinction and that the principal factor involved is the change of surface area, an assumption implicit in all work on mitochondrial swelling and probably justified where, as in the present case, the mitochondria exist in spherical form.) No difference in size of mitochondria from normal and hormone-treated animals was found. The results appear to suggest therefore that mitochondria isolated from growth-hormone-treated rats are normal at least with respect to size and swelling properties.

Mitochondrial permeability and oxidative phosphorylation in vitro. It was also possible to demonstrate that mitochondria in which swelling had been induced *in vitro*, by the preincubation of mitochondria from normal rat livers with growth hormone for periods up to 4 hr. at 0° or up to 40 min. at 27°, had the same P/O ratio as did the mitochondria preincubated in the same way but in the absence of growth hormone (the values at 0° for 4 hr. were: controls 1·05; treated animals 1·10; three animals were used in each group). Addition of growth hormone *in vitro* to digitonin particles from normal rats was also without effect on the measured P/O ratio.

Pyridine nucleotide content of the isolated mitochondria. The concentration of oxidized and reduced DPN was determined in the mitochondria from normal rats and in the mitochondria from rats treated 4 hr. previously with growth hormone. The results are shown in Table 2. There is a statistically significant increase in both the total mitochondrial DPN and DPNH and in the total diphosphopyridine nucleotide expressed per gram of liver. The ratio of DPN to DPNH is also very different in the two groups of animals. In the whole liver the ratio DPN to DPNH usually found is of the order of 2 (Holzer, Goldschmidt, Lamprecht & Helmreich, 1954; Glock & McLean, 1957), but the ratio found here in mitochondria appears to be considerably higher than this. In mitochondria from growth-hormone-treated animals there is a greater proportionate increase in the DPNH than in the DPN. These findings parallel those described for the whole liver by Greenbaum & Graymore (1956). The possibility that the increased nucleotide content of the growth-hormone-treated mitochondria was due to the release of bound forms of the type described by Purvis (1958) was tested by preincubating the mitochondria of normal and hormone-treated animals with ADP for 7 min. at 30° as described by Purvis (1958). No increase in the measured pyridine nucleotide was found in either set of mitochondria.

DISCUSSION

The injection of pituitary growth hormone into normal intact rats causes a decrease in the efficiency of oxidative phosphorylation of liver mitochondria oxidizing L-β-hydroxybutyrate.

The lowered efficiency has been demonstrated in rats after the injection of 1 mg. of the hormone, an amount which raises the blood concentration by about 0·1 μM. The normal physiological concentration of the hormone in blood has been estimated by Dr J. H. Ottaway (personal communication) to be about 0·02 μM. Thus the amount injected is within the physiological range.

Several facts point to the site of action of growth hormone as being at the level of the mitochondrial

Table 2. *Amounts of oxidized and reduced diphosphopyridine nucleotide in mitochondria from the livers of normal and growth-hormone-treated rats*

Treatment	No. of animals	Wt. of liver (g.)	Total DPN (μg.)	Total DPNH (μg.)	Total DPN+DPNH (μg.)	Total mitochondrial DPN+DPNH/g. of liver (μg.)	DPN/DPNH
Controls	6	6·54±0·12	175±7·1	41±5·4	216±8·7	32·9±1·3	4·82±0·73
Growth-hormone treated	6	6·27±0·28	234±16·4	117±8·2	351±21·4	55·7±4·1	2·03±0·17
P (controls v. hormone-treated)		0·442	0·009	< 0·001	< 0·001	0·001	0·005

membrane. First is the finding that although the P/O ratios in mitochondria isolated from normal and growth-hormone-treated animals differ, the P/O ratios in digitonin particles prepared from these mitochondria do not. Secondly, there is the activity of growth hormone as a swelling agent. Since the increased size of the mitochondria is due to the slow penetration of sucrose under the action of the swelling agent, swelling can be equated with increased mitochondrial permeability (Tedeschi & Harris, 1955, 1958). On a molar basis growth hormone seems to be at least as active as thyroxine and, further, there appears to be a close correlation between the growth-promoting activity *in vivo* and the mitochondrial-swelling activity *in vitro*. Thirdly, the idea that many hormones may have a common mechanism of action is an attractive one. In addition to growth hormone, swelling can be induced by insulin and adrenocorticotrophic hormone (although these two hormones were less effective than growth hormone). The addition of the appropriate amount of zinc (60μg.) did not reproduce the insulin effect. In addition to the above-mentioned hormones thyroxine (Martius & Hess, 1955) and hydrocortisone (Gallagher, 1960) share this property. Prolactin failed to induce swelling in liver mitochondria but it is possible that it would have been more effective if tested on the mitochondria of the mammary gland, its normal target organ. All the other proteins tested stabilized mitochondria against spontaneous swelling, or even against the action of hormones which would have produced swelling in their absence. All these facts point to a similarity in the mode of action of growth hormone and thyroxine.

However, some of the results obtained lead to the opposite conclusion. First, growth hormone does not uncouple oxidative phosphorylation *in vitro*, whereas this has been clearly demonstrated for thyroxine (Lehninger, Ray & Schneider, 1959; Martius & Hess, 1955). In view of the suggestion that growth hormone may be broken down to an active form *in vivo* (Park, Brown, Cornblath, Daughaday & Krahl, 1952; Bornstein, 1953) it might be held that protein growth hormone, as used here, is unsuitable for studies *in vitro*. It should be noted, however, that the intact protein hormone produces an immediate effect *in vitro* on mitochondrial permeability although it does not uncouple under identical conditions. Even increasing the level of hormone up to ten times that normally used has been found to be ineffective in changing the P/O ratio. Secondly, it was found that there was a significant fall in the P/O ratio of the mitochondria isolated from growth-hormone-treated rats, although it was not possible to demonstrate any gross changes in permeability as measured either by their subsequent response to the addition of either growth hormone or thyroxine to the suspending medium, or by their size. Thirdly, no loss of pyridine nucleotides could be detected. Maley & Lardy (1955) have reported that there is a loss of up to 40% of the total pyridine nucleotides from the mitochondria of hyperthyroid rats, and Lehninger *et al.* (1959) found a loss of bound DPN from the interior of the mitochondria of thyroxine-treated rats. These latter authors believe that this loss could account for the lowered P/O ratios found in the mitochondria of such treated animals. This explanation does not appear to apply to growth hormone. As can be seen from Table 2 there is no loss of DPN from the mitochondria of the growth-hormone-treated rats; on the contrary, they probably contain more pyridine nucleotide than the controls. Whether this increase represents an increased synthesis or whether it is due to the liberation of a bound form of the type suggested by Purvis (1958), either by the hormone or by ADP, is not clear, but this latter alternative is made somewhat unlikely in view of the fact that preincubation with ADP made no difference to the amount of extractable DPN from either normal or growth-hormone-treated mitochondria, a result in keeping with those of Klingenberg & Slenczka (1959). The increases found in the mitochondria in the present study show a similar trend to those found in the whole liver of growth-hormone-treated rats (Greenbaum & Graymore, 1956), in that there is a greater increase in the DPNH than the DPN, with a consequent fall in the DPN/DPNH ratio.

A final difference between the action of growth hormone and thyroxine should be noted. The decrease in the P/O ratio caused by growth hormone is due to a 40% increase in the rate of oxidation with no increase in the associated phosphorylation (Table 1), whereas the uncoupling produced by thyroxine is characterized by a small increase in the rate of oxidation associated with a fall in the accompanying phosphorylation. This difference would also appear to indicate a significant difference in the way the two hormones produce their effect. It seems unlikely that the growth-hormone effect can be ascribed to a hormonally induced release of the 'R' factor of Remmert & Lehninger (1959), since this increases both respiration and phosphorylation, i.e. no change in P/O ratio, whereas other agents such as the 'U' factor (Lehninger & Remmert, 1959) and mitochrome (Polis & Shmukler, 1957) both lower the P/O ratio by decreasing the rate of phosphorylation. The increased rate of oxidation of β-hydroxybutyrate found here is of particular interest since it will be seen from Table 1 that when the mitochondrial-permeability barrier is circumvented by using digitonin particles the rate of β-hydroxybutyrate

oxidation is the same in mitochondrial fragments from normal and growth-hormone-treated rats, and, further, that both are at the same level as the enhanced value found in intact mitochondria from growth-hormone-treated animals (calculating the values for the intact mitochondria and for digitonin particles in terms of equivalent quantity of phosphorylation). This would seem to indicate the removal of an inhibition and points to the possibility that growth hormone increases the rate of penetration of the substrate. The unchanged rate of phosphorylation in all these preparations may indicate that the lowered P/O ratio found is not so much due to an uncoupling action as to the stimulation of a non-phosphorylating pathway.

The physiological significance of these findings is difficult to evaluate as a lowered efficiency of oxidative phosphorylation would be of doubtful advantage to a growing animal. It is, however, possible that, as Ernster (1956) has suggested, swelling can be regarded as a process which confers a physiological advantage on an animal. A reversible swelling of the mitochondria might be a regulatory process controlling the transfer of substrates and other materials between mitochondrion and cytoplasm. Regulation of metabolic processes by changes in permeability could well be one of the main mechanisms of hormonal activity.

SUMMARY

1. The efficiency of oxidative phosphorylation in liver mitochondria from normal and growth-hormone-treated rats has been measured with L-β-hydroxybutyrate, reduced diphosphopyridine nucleotide or glutamate as substrate. There is a decrease in the efficiency when L-β-hydroxybutyrate is used but not when reduced diphosphopyridine nucleotide or glutamate is substrate. The decreased P/O value is due to an increased rate of oxidation with no change in the rate of phosphorylation.

2. There was no difference in the P/O ratio, with L-β-hydroxybutyrate as substrate, when digitonin particles isolated from normal and growth-hormone-treated animals were compared.

3. Growth hormone at physiological concentrations produced striking increases in mitochondrial permeability when added to mitochondria suspended in sucrose.

4. The possibility that the effect of growth hormone on oxidative phosphorylation is secondary to an effect on mitochondrial permeability has been considered and the possible relationship between the effects of growth hormone and of thyroxine are discussed.

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Some Studies on the Occurrence of Sialic Acid in Human Cartilage

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Most studies on cartilage have involved investigations of the acid mucopolysaccharide components. There is evidence to suggest, however, that cartilage also contains significant amounts of high-molecular-weight neutral polysaccharide material that does not contain hexuronic acid or sulphate (Hisamura, 1938; Glegg, Eidinger & Leblond, 1954; Glegg & Eidinger, 1955; Herring & Kent, 1958).

In recent years there has been a growing interest in a family of compounds consisting of acylated neuraminic acids, the so-called 'sialic acids', first demonstrated by Blix (1936) in submaxillary gland. Compounds containing sialic acid, usually termed 'glycoproteins', have since been found in a wide variety of mammalian organs and secretions (for a review see Zilliken & Whitehouse, 1958) but few reports are available on the glycoproteins in connective tissue. Sialic acid has been detected in gelatin prepared from mammalian collagen (Courts, 1959, 1960), in the epiphyseal cartilage of young rabbits (Castellani, Ferri, Bolognani & Graziano, 1960) and in various other animal tissues by a histochemical technique (Diezel, 1955). No reports are available, however, on similar components in cartilage from human sources.

In view of the fact that sialic acid-containing gycoproteins possess a wide range of biological activities (for a review see Bettelheim-Jevons, 1958) and appear to play a part in many disease processes (Greenspan, 1955; Lockey, Anderson & Maclagan, 1956), the presence of these compounds in human cartilage has been investigated.

In the present work it soon became apparent that fractions prepared from human cartilage under mildly alkaline conditions gave strong positive reactions for sialic acid in the diphenylamine test (Dische, 1929). Since many methods of obtaining chondroitin sulphate involve a preliminary mild alkaline extraction of cartilage it would appear that, initially at least, such chondroitin sulphate preparations are contaminated with components containing sialic acid, in addition to peptide material.

It is now fairly well established that the difficulties in preparing protein-free chondroitin sulphate are due to the presence of relatively stable protein–mucopolysaccharide complexes. Small amounts of these complexes can be extracted from bovine cartilage under neutral conditions (Shatton & Schubert, 1954; Muir, 1958) and larger amounts under mildly alkaline conditions (Partridge & Davis, 1958). Subsequent purification of mucopolysaccharides relies either on treatment with proteolytic enzymes (Webber & Bayley, 1956) or the use of relatively strong alkali or acid (Einbinder & Schubert, 1950) in order to split the protein–mucopolysaccharide bonds. The resulting protein-free mucopolysaccharide, however, is generally of a low molecular weight (Mathews & Dorfman, 1953; Mathews, 1956).

In view of the presence of sialic acid in human cartilage the question arose whether it was present in the form of sialoproteins similar to those in human urine (Anderson & Maclagan, 1955) and serum (Winzler, 1955), or whether it was connected in some way with the mucopolysaccharide–protein complexes of the type first reported by Shatton & Schubert (1954). It therefore appeared of interest to determine whether the difficulties attending the removal of peptide material from chondroitin sulphate preparations applied also to the removal of the sialic acid-containing components.

Three fractions have therefore been extracted from human cartilage: a major fraction at pH 10·6 and two smaller fractions at pH 11·6. The major fraction has been subjected to chemical and enzymic procedures designed to remove both protein and sialic acid-containing components. In the light of the results obtained possible relationships between sialic acid and the mucopolysaccharides present in human cartilage are discussed. A preliminary report of this part of the work has been published (Anderson, 1960).

METHODS AND MATERIALS

Chondroitin sulphate (sodium salt), prepared from bovine nasal septa (Analysis; N, 3·6; S, 2·4; ash, 7·2%), was bought from Mann Research Laboratories (New York); orosomucoid and *N*-acetylneuraminic acid were gifts from Dr R. J. Winzler.

The following three complexes were gifts from Dr H. Muir. Chondroitin sulphate A–peptide complex isolated

from pig tracheal cartilage (Muir, 1958). Chondroitin sulphate A from human aorta, isolated by Dr Muir as follows: the tissue was digested with pepsin (pH 2) and then with pancreatin (pH 7). The sulphated polysaccharides were purified by precipitating twice with 2-aminoacridine–HCl and then with ethanol. The chondroitin sulphate A was separated from the other polysaccharides by passage through a Celite–cellulose column, and elution with zinc acetate–propanol (Berenson, 1958). It was then converted into the calcium salt, precipitated with 40% (v/v) ethanol, reprecipitated with 2-aminoacridine–HCl and reisolated as the sodium salt. Chondroitin sulphate B, from human aorta, was isolated by Dr Muir in the same way as described for chondroitin sulphate A above as far as the Berenson procedure. After elution from the column it was separated from the other polysaccharides by glass-fibre electrophoresis at pH 7·3. Both chondroitin sulphates A and B from human aorta contained peptide fragments still intact after ultrafiltration, consisting chiefly of glycine and serine together with smaller amounts of glutamic acid and aspartic acid and traces of some other amino acids. The proportions of these amino acids differed in the two preparations.

Total nitrogen. Solutions containing 0·3–0·4 mg. of N were digested with 1·5 ml. of 1% (w/v) selenium in 50% (v/v) H_2SO_4 for 4 hr. N was determined by a modified nesslerization method (Koch & McMeekin, 1924).

Total hexose. The orcinol method, as described by Winzler (1955), was employed, equal amounts, by weight, of D-galactose and D-mannose being used as the standard.

Sulphate. A modification of the benzidine method of Dodgson & Spencer (1953) was used. Digestion of solutions containing 0·1–0·2 mg. of sulphate was carried out for 4 hr. in a mixture of conc. HNO_3 (1 ml.) and perchloric acid (sp.gr. 1·54; 1 ml.). After neutralization and adjustment of the digest volume to 10 ml. with water, 1 ml. was taken for assay. The intensity of an appropriate dilution of the final coloured product was measured in 1 cm. cells in a Unicam SP. 500 spectrophotometer at 500 mμ.

Hexosamine. The method of Elson & Morgan (1933) was used, as described by Winzler (1955), employing a standard of α-D-glucosamine–HCl, assay figures being expressed in terms of the free base. Hydrolysis was carried out in 3N-HCl for 6 hr. at 100°.

Hexuronic acid. The carbazole method of Dische (1955) was used, with glucuronic acid as the standard.

Protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was employed, with reconstituted freeze-dried serum (Glaxo Laboratories Ltd., Greenford) of known protein content as the standard.

Sialic acid. The acid diphenylamine reaction of Dische (1929) was employed, as used by Anderson & Maclagan (1955). The test solution (1 ml.) was heated at 100° for 30 min. with a diphenylamine solution (2 ml.) consisting of 1 g. of diphenylamine (recrystallized twice from ethanol–water) dissolved in a mixture of conc. H_2SO_4 (A.R.; 10 ml.) and acetic acid (A.R.; 90 ml.). Absorption curves over the range 450–850 mμ were determined with 1 cm. cells in the spectrophotometer. *N*-Acetylneuraminic acid and human serum orosomucoid (containing 15% of *N*-acetylneuraminic acid) were used as standards. Sialic acid values (in terms of *N*-acetylneuraminic acid) were calculated from the differences in extinctions at 525 mμ in the presence or absence of diphenylamine in order to correct for non-specific colour development.

Confirmatory evidence for the presence of sialic acid was obtained by the method developed by Svennerholm (1958). The test sample (10–30 mg.) was heated with 0·1N-H_2SO_4 (80°, 1 hr.), the hydrolysate was centrifuged and the supernatant fluid passed first through Dowex-50 × 8 (200–400 mesh, H^+ form) and then through Dowex-1 × 8 (100–200 mesh, acetate form). Sialic acid was eluted from Dowex-1 with an acetate buffer (1M, pH 4·6), the first 15 ml. of effluent being collected. Sialic acid was determined as follows. A portion (1 ml.) of effluent was heated at 100° for 15 min. with 2 ml. of resorcinol reagent (0·2 g. of resorcinol, A.R.) dissolved in a mixture of conc. HCl (A.R.; 80 ml.), water (20 ml.) and 0·1M-Cu_2SO_4 (0·25 ml.). The cooled digestion mixture was then shaken with *iso*amyl alcohol (5 ml.). Absorption curves of the chromogens extracted into the alcohol phase were determined over the range 450–750 mμ in the spectrophotometer. *N*-Acetylneuraminic acid, carried through the same procedure, was used as a standard.

Amino acids. The two-dimensional paper-chromatographic technique as modified by Bowden (1959) was used, employing the phenol solution of Datta, Dent & Harris (1950) as the first solvent and the ethyl methyl ketone–butanol–di*cyclo*hexylamine–water mixture of Hardy, Holland & Naylor (1955) as the second solvent. The second solvent was found to give a cloudy solution and was therefore modified to a composition of ethyl methyl ketone (110 ml.), butanol (110 ml.), di*cyclo*hexylamine (20 ml.) and water (50 ml.). This solvent system enabled amino acids to be identified not only by position but also by colour, after development of the chromatogram with ninhydrin. Hydrolysis of the fractions obtained from cartilage (see below) was carried out in stoppered tubes with 6N-HCl for 8 hr. at 100° in an oven. Excess of acid was removed *in vacuo* over solid NaOH.

Tissue preparations

Disintegration of cartilage. Human cartilage, from a mixture obtained from articular (28%), semilunar (12%), costal (28%) and tracheal (32%) sources, was collected from post-mortem cases restricted to subjects under 65 years, where death had resulted either from accident or pathological conditions unrelated to joint or cartilage diseases. After removal of adhering tissue and bone, the cartilage was washed briefly several times in 0·9% NaCl solution until white and stored frozen until used. To avoid the difficulties of homogenizing for long periods of time in large volumes of extracting solutions, the cartilage was first disintegrated in a mill (developed for crushing whole bone by Dr J. T. Scales, Department of Biomechanics and Surgical Materials, Institute of Orthopaedics, in association with Christy and Norris Ltd., Chelmsford, and British Oxygen Research and Development Ltd., London), in which the grinding chamber was surrounded by a jacket containing liquid nitrogen. By this means the cartilage was disintegrated into particles, less than 1·6 mm. in diameter, which readily dispersed in the aqueous solvents used for the extraction.

Fractionation of cartilage. Preliminary experiments were carried out to determine the effect of pH on the amount of material that could be extracted from the disintegrated

cartilage. All operations were performed at 4°. A known amount of cartilage was extracted successively with three equal volumes of a solution of known pH. Each extraction was performed by mechanical stirring overnight, followed by centrifuging. The three supernatant fluids obtained were combined, filtered through Whatman no. 1 filter paper and the pH was determined. Fractionation of the supernatant fluid was then carried out by the addition of ethanol solution [95% ethanol containing 1% (w/v) of potassium acetate and 1% (v/v) of acetic acid] in the following manner. After adjustment of the supernatant fluid to pH 7 with acetic acid, ethanol solution (2 vol.) was slowly added with constant stirring (final ethanol concentration 63%, v/v). After standing overnight the flocculent precipitate was centrifuged down and the supernatant fluid (S) retained. The precipitate was suspended in water and brought into solution by addition of alkali to pH 7. Reprecipitation was carried out by the addition of ethanol solution (2 vol.) and the product (fraction 1) washed with ethanol and ether and dried *in vacuo*. To the supernatant fluid (S) a further 2 vol. of ethanol solution was added (final ethanol concentration 84%). The flocculent precipitate, after standing overnight, was treated in the same manner as for fraction 1. The resulting product was termed fraction 2. Details of the yields of fractions 1 and 2, obtained at three different pH values, are shown in Table 1.

In view of these preliminary experiments the following method of bulk extraction of the cartilage was employed. Portions (50 g.) of the disintegrated cartilage were suspended in a glycine buffer (0·1M, pH 11·5, 250 ml.) and stirred mechanically for 24 hr. The residue of cartilage remaining was centrifuged down, the supernatant fluid adjusted to pH 7 and retained, and the extraction was repeated twice more on the residue with the same volume of glycine buffer each time. The three neutral supernatant fluids were combined, filtered and ethanol solution (2 vol.) was added (final ethanol concentration 63%). After standing overnight the precipitate was centrifuged down, suspended in water (150 ml.) and brought into solution by adjustment to pH 7 with alkali. After dialysis of the solution against distilled water for 5 days with frequent changes, ethanol solution (2 vol.) was added. After standing overnight the precipitate was collected by centrifuging, washed with ethanol and ether and dried *in vacuo*. This product was termed fraction A.

The cartilage residue obtained after the three extractions with glycine buffer was then suspended in 0·5N-NaOH (250 ml.) and stirred mechanically overnight. After centrifuging, the supernatant fluid was adjusted to pH 7 and retained. The residue was extracted once more with the same volume of 0·5N-NaOH. The two neutral supernatant fluids were combined and ethanol solution (2 vol.) was added (final ethanol concentration 63%, v/v). After standing overnight the precipitate was centrifuged down and the supernatant fluid (S) retained. The precipitate was suspended in water (150 ml.), the mixture was adjusted to pH 7 and the resulting solution dialysed against water for 5 days with frequent changes. Reprecipitation was then carried out by the addition of ethanol solution (2 vol.) and the precipitate collected by centrifuging after standing overnight. After washing with ethanol and ether it was dried *in vacuo*. This product was termed fraction B_1. To the supernatant fluid (S) was added 2 vol. of ethanol solution (final ethanol concentration 84%, v/v). After standing overnight the precipitate was centrifuged down and the residue suspended in water (60 ml.). After adjustment to pH 7 the solution was dialysed and reprecipitation carried out as for fraction B_1, except that ethanol solution was added to a final concentration of 84% (v/v). This product was termed fraction B_2. The yields of these three fractions are shown in Table 2.

EXPERIMENTAL AND RESULTS

Specificities of the diphenylamine and resorcinol reactions for sialic acid in the presence of protein and acid mucopolysaccharide components

Diphenylamine reaction. Fraction A gave a violet colour when heated with the diphenylamine reagent. Fractions B_1 and B_2 gave violet–blue colours. The absorption curves of the chromogens formed by these three fractions are shown in Fig. 1, after subtraction of the extinctions produced by a reagent blank consisting of water (1 ml.) in place of the test solution (1 ml.). The absorption curves of the light-blue chromogens formed with a sample of chondroitin sulphate, a chondroitin sulphate A–protein complex isolated from pig tracheal cartilage (Muir, 1958) and glucuronic acid are shown in Fig. 2. The absorption curves of the violet chromogens formed with *N*-acetylneuraminic acid and human-serum orosomucoid are shown for comparison in Fig. 2. L-Fucose (0·2 mg.), α-D-glucosamine–HCl (0·3 mg.), bovine-serum albumin (2 mg.), serine (0·2 mg.), glycine (0·2 mg.), aspartic acid (0·2 mg.), glutamic acid (0·2 mg.) and an equal mixture, by weight, of D-galactose and D-mannose (0·2 mg.), gave no visible colours with the diphenylamine reagent.

The percentages of sialic acid in cartilage fractions A, B_1 and B_2 are shown in Table 2, together with the results of analysis for other constituents. The sialic acid contents of the fractions extracted at three different pH values are shown in Table 1.

Mild acid hydrolysis of fraction A. Fraction A (20 mg.) was dissolved in 0·01N-HCl (6 ml.) and heated at 80° for 1 hr. A control, consisting of fraction A (20 mg.) in water (6 ml.), was treated similarly. Each solution was then dialysed against distilled water (10 ml.) for 24 hr., followed by a second dialysis against a further 10 ml. of water for the same period. The two diffusates obtained from the control gave no reaction when heated with the diphenylamine reagent. The two diffusates from the acid-hydrolysed fraction A, when caused to react quantitatively with the diphenylamine reagent, showed that 93% of the total sialic acid present initially in fraction A had diffused out in 48 hr. The absorption curves of the chromogens formed by these two diffusates are shown in Fig. 3.

The difference in the relative heights of the absorption maxima at 525 and 650 mμ shown by the first diffusate (Fig. 3) and that shown by authentic *N*-acetylneuraminic acid (Fig. 2) was probably due to the presence of the small amount of monosaccharides liberated from fraction A by mild acid hydrolysis. Courts (1959) has shown that some monosaccharides form chromogens with diphenylamine which absorb strongly at 650 mμ. Fructose and deoxyribose, which both form chromogens with diphenylamine absorbing at 650 mμ, were absent from the diffusate, however, since heating the diffusate with the resorcinol reagent (see below) formed a chromogen identical with that produced by authentic *N*-acetylneuraminic acid. Both fructose and deoxyribose form chromogens

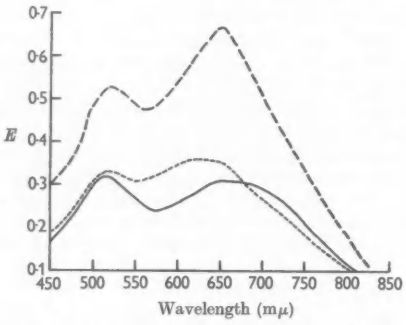

Fig. 1. Absorption curves of the chromogens obtained on heating three fractions (2 mg.) from human cartilage with the diphenylamine reagent: ——, fraction A; - - - -, fraction B_1; — — —, fraction B_2.

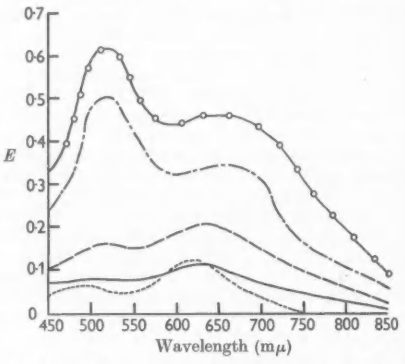

Fig. 2. Absorption curves of the chromogens obtained on heating the diphenylamine reagent with: ——, chondroitin sulphate (1 mg.); - - - -, glucuronic acid (0·3 mg.); — — —, chondroitin sulphate A–peptide complex (2 mg.) (isolated from pig tracheal cartilage; Muir, 1958); —·—·—, *N*-acetylneuraminic acid (0·1 mg.); —o—o—, human-serum orosomucoid (1 mg.).

Table 1. *Extraction of human cartilage at three different* pH *values*

Amount of wet disintegrated cartilage extracted (g.)	Extraction medium*	pH of combined extracts	Yield (mg./100 g. of wet cartilage) Fraction 1†	Fraction 2‡	Sialic acid content of fraction 1 (%)
30	0·5N-NaOH (100 ml.)	11·6	4100	320	3·6
10	Glycine buffer (0·1M, pH 11·5, 50 ml.)	10·6	2230	70	3·7
20	Glycine buffer (0·1M, pH 9·5, 50 ml.)	9·0	563	Trace	3·7

* The cartilage was extracted three times with the extraction medium and the extracts were combined.
† Obtained by precipitation of the combined extracts with ethanol solution (final ethanol concentration 63%, v/v).
‡ Obtained by ethanol precipitation of the supernatant fluid remaining after removal of fraction 1 (final ethanol concentration 84%, v/v).

Table 2. *Yields and analysis of fractions A, B_1 and B_2 from human cartilage*

Fraction A was obtained from cartilage by extraction at pH 10·6 followed by ethanol precipitation (63%, v/v). Fractions B_1 and B_2 were obtained by extraction of the remaining cartilage at pH 11·6, followed by ethanol precipitation to 63% (v/v) and 84% (v/v) respectively. Protein was (1) determined by the method of Lowry, Rosebrough, Farr & Randall (1951), or was (2) calculated by multiplying total N by 6·25 after subtracting hexosamine N (7·8%) and sialic acid N (4·5%).

Fraction	Yield (mg./100 g. of wet cartilage)	N (%)	Protein (%) (1)	(2)	Sialic acid (%)	Total hexose (%)	Hexosamine (%)	Hexuronic acid (%)	Sulphate (%)
A	2915	8·3	34·0	46·0	3·7	14·6	10·9	8·6	10·7
B_1	608	9·2	42·3	53·0	4·1	14·0	6·3	7·3	7·7
B_2	151	9·5	43·3	55·0	6·4	11·6	6·5	0·3	5·6

with the resorcinol reagent which absorb strongly in the region 450–500 mμ.

The acid-hydrolysed fraction A, after prolonged dialysis against water, gave a colourless product when heated with the diphenylamine reagent (Fig. 3). The analysis for native fraction A, and for fraction A after acid hydrolysis followed by prolonged dialysis against water, is shown in Table 3.

Resorcinol reaction. Fraction A formed a brown chromogen, the absorption curve of which is shown in Fig. 4. Fractions B_1 and B_2 behaved similarly. The high absorption in the region 450–500 mμ was found to be due to the hexose and hexuronic acid constituents in the fractions, both of which form orange–yellow chromogens with resorcinol. The following substances were found to give negative reactions: α-D-glucosamine–HCl (0·1 mg.), serine (0·2 mg.), glycine (0·2 mg.), glutamic acid (0·2 mg.), aspartic acid (0·2 mg.). The chromogen produced with *N*-acetylneuraminic acid gave a strong absorption at 575 mμ, with an inflexion in the curve at 612 mμ (Fig. 4).

After the following pretreatments fractions A, B_1 and B_2 formed blue chromogens with the resorcinol reagent with absorption spectra identical with that produced by *N*-acetylneuraminic acid: (*a*) mild acid hydrolysis followed by dialysis (for experimental details see above); (*b*) treatment by the procedure described by Svennerholm (1958) (see under Methods and Materials; the absorption curve of the chromogen produced with fraction A after this treatment is shown in Fig. 4).

These results indicate that the resorcinol reaction, unlike the diphenylamine reaction, can be used for estimating sialic acid only after removal of interfering hexoses and hexuronic acid.

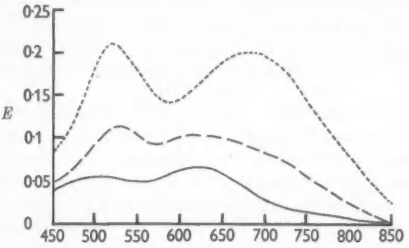

Fig. 3. Absorption curves of the chromogens obtained on heating the diphenylamine reagent with: ——, fraction A (2 mg.) after acid hydrolysis (0·01 N-HCl, 80°, 1 hr.) and prolonged dialysis against water; - - - -, the first diffusate (1 ml.) obtained from acid-hydrolysed fraction A after dialysis against 10 ml. of water during the period 0–24 hr.; — — —, the second diffusate (1 ml.), obtained from acid-hydrolysed fraction A after dialysis against a further 10 ml. of water during the period 24–48 hr.

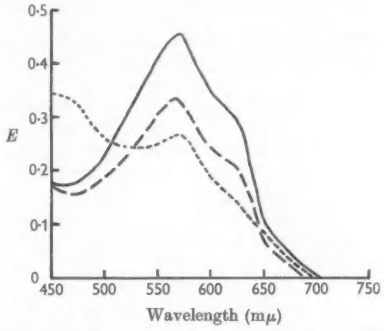

Fig. 4. Absorption curves of the chromogens obtained on heating the resorcinol reagent with: ——, *N*-acetylneuraminic acid (0·1 mg.); - - - -, fraction A (2 mg.); — — —, fraction A after acid hydrolysis (0·1 N-H_2SO_4, 80°, 1 hr.) followed by purification of sialic acid by the ion-exchange method of Svennerholm (1958).

Table 3. *Estimation of constituents in fraction A (20 mg.) before mild acid hydrolysis and after mild acid hydrolysis followed by prolonged dialysis against water*

Hydrolysis of fraction A was carried out with HCl (0·01 N, 80°, 1 hr.).

Constituent	Before hydrolysis (mg.)	After hydrolysis and prolonged dialysis (mg.)	Decrease after hydrolysis and dialysis (%)
Hexuronic acid	1·7	1·7	0
Hexosamine	2·1	2·1	0
Protein*	6·8	6·0	12
Total hexose	2·9	2·6	10
Sialic acid	0·7	0	100
Complete hydrolysis (5 N-HCl, 100°, 6 hr.)	Large amount of humin	Small amount of humin	—
Solubility in water	Complete	Partial	—

* Method of Lowry *et al.* (1951).

Amino acid composition of fractions A, B_1 and B_2

Chromatography of 1 mg. of the hydrolysed fractions revealed the presence of aspartic acid, glutamic acid, glycine, alanine, serine, threonine, valine, leucine (isoleucine), arginine, lysine, phenylalanine, tyrosine, proline, hydroxyproline and phosphoserine. In addition, a strong spot was detected in the position corresponding to hexosamine. Glucosamine and galactosamine were not separated with the solvent system employed. Chromatography of 0·5 mg. of the hydrolysed fractions revealed the presence of the same amino acids in all three fractions, except that in fractions A and B_2 no hydroxyproline could be detected. One unidentified component reacting with ninhydrin was present in fraction A and two unknown components were in fractions B_1 and B_2.

Non-enzymic fractionation procedures on fraction A

Deproteinizing procedures. The procedure used by Webber & Bayley (1956) to decrease the N content of chondroitin sulphate preparations was followed. Fraction A (100 mg.) was dissolved in water (20 ml.) and 5 ml. of a solution of 5% (w/v) phosphotungstic acid in 2N-HCl added. The precipitate that formed was centrifuged down and the supernatant fluid (S) retained. The precipitate was suspended in water (20 ml.) and brought into solution by adjustment to pH 7 with alkali. After prolonged dialysis against water the precipitate was recovered by addition of ethanol solution (2 vol.), followed by washing with ethanol and ether. The product (R_1) was dried *in vacuo*. The supernatant fluid (S) was neutralized with alkali and freed of phosphotungstic acid by dialysis. Ethanol solution (2 vol.) was added to the dialysed solution and the precipitate (R_2) was washed with ethanol and ether and dried *in vacuo*. The yields and analysis of R_1 and R_2 are given in Table 4. A control experiment showed that chondroitin sulphate was completely soluble in the phosphotungstic acid reagent.

Although a large proportion of the mucopolysaccharides in fraction A was concentrated in the R_2 fraction, a considerable amount of material which contained sialic acid was also present. Treatment of R_2 with trypsin, by the method of Shatton & Schubert (1954), degraded 34% of the peptide material to dialysable fragments but did not result in the liberation of dialysable sialic acid. Attempts to adsorb the sialic acid-containing components of R_2 with Lloyd's reagent (amorphous aluminium silicate) by the method of Pearce & Watson (1949) were unsuccessful.

Similarly, although shaking an aqueous solution of fraction A with chloroform–amyl alcohol (Sevag, 1934) removed 22% of the fraction in the form of insoluble material, the remainder still contained both hexuronic acid and sialic acid.

Ammonium sulphate fractionation. Fraction A (100 mg.) was dissolved in water (20 ml.) and 20 ml. of a sat. aq. solution of $(NH_4)_2SO_4$ was added. After standing 6 hr. at 4° the mixture was centrifuged and the precipitate (R_1) treated as described below. Solid $(NH_4)_2SO_4$ was added to the supernatant fluid until fully saturated and the mixture centrifuged after standing overnight. The residue (R_2) was treated as described below. The supernatant fluid was dialysed against distilled water, solid NaCl was added to 0·2 M and the solution poured into acetone (5 vol.). After standing overnight the precipitate (R_3) was centrifuged down. R_1, R_2 and R_3 were each dissolved in water (20 ml.) at pH 9 and the solutions were dialysed against distilled water. Reprecipitation was carried out with NaCl and acetone as described above, the products being washed with ethanol and ether and dried *in vacuo*. Yields and analysis of the three fractions are shown in Table 5. A control experiment showed that chondroitin sulphate was completely soluble in sat. aq. $(NH_4)_2SO_4$.

Adsorption on to benzoic acid. The method of Anderson & Maclagan (1955) for the isolation of the sialic acid-containing glycoproteins in urine was used. All operations were performed at 4°. Fraction A (50 mg.) was dissolved in water (500 ml.). 2M-Sodium benzoate (62·5 ml.) was added, followed by the slow addition with constant stirring of

Table 4. *Phosphotungstic acid precipitation of fraction A*

Fraction A (100 mg.) was treated with a solution of phosphotungstic acid in 2N-HCl (5%, w/v). R_1 and R_2 represent the insoluble and soluble fractions respectively.

Component	Yield (mg.)	Sialic acid (%)	Hexuronic acid (%)	Protein* (%)
R_1	38	1·9	2·5	50·0
R_2	64	4·3	11·9	24·0

* By the method of Lowry *et al.* (1951).

Table 5. *Yields and analysis of the residues obtained from fraction A (100 mg.) after ammonium sulphate fractionation*

R_1 and R_2 were the fractions insoluble at half and full saturation with $(NH_4)_2SO_4$ respectively. R_3 was the residue soluble in fully saturated $(NH_4)_2SO_4$ solution.

Fraction	Yield (mg.)	Sialic acid (%)	Hexuronic acid (%)	Protein* (%)
R_1	21	3·8	2·8	37·6
R_2	65	4·1	9·0	31·8
R_3	16	0·3	11·7	3·4

* By the method of Lowry *et al.* (1951).

2N-HCl (50 ml.). After standing for 1 hr. the benzoic acid precipitate was filtered off under suction, drained thoroughly and treated with acetone (100 ml.). The residue remaining was centrifuged down, washed once with acetone and suspended in water (25 ml.). The residue dissolved completely after adjustment to pH 9 with alkali. Reprecipitation was carried out by addition of solid NaCl, to 0·2M, and acetone (5 vol.). The precipitate was dialysed against distilled water and reprecipitated in the same manner. The yield, after washing with ethanol and ether and drying *in vacuo*, was 34 mg. (Analysis: sialic acid, 3·7; hexuronic acid, 6·0; protein, 34%). The characteristics of the absorption curve of the chromogen formed by this product with the diphenylamine reagent were identical with the curve shown in Fig. 1 for fraction A.

A control experiment with a sample of chondroitin sulphate (50 mg.) showed that less than 2% was adsorbed on to benzoic acid. The remainder could be recovered from the benzoic acid filtrate on addition of ethanol solution (2 vol.).

Enzymic fractionation procedures on fraction A

Papain digestion. Crude papain (100 mg.) was activated by incubating at 45° for 30 min. in an acetate buffer (0·02M; pH 5·4; 10 ml.) containing 0·01M-cysteine. After filtering off the insoluble material, 1 ml. of filtrate was added to fraction A (100 mg.) contained in the acetate buffer (5 ml.). The mixture was incubated overnight at 45°. A control, consisting of a mixture of 1 ml. of papain filtrate in 5 ml. of acetate buffer, was treated similarly. Each mixture was then dialysed against water and the non-dialysable components were precipitated by the addition of solid NaCl (to 0·2M) and ethanol solution (3 vol.). The precipitates were washed with ethanol and ether and dried *in vacuo*. Fraction A, after digestion, yielded 61 mg. The residue from the control experiment was too slight to collect and estimations on this residue were performed on portions after dissolving the residue in water (10 ml.) [Analysis for fraction A after digestion (corrected for the papain control): sialic acid, 4·7; hexuronic acid, 15·2; protein, 14·0%]. The amino acid composition was similar to that of native fraction A, except that no phosphoserine could be detected.

Hyaluronidase digestion. Fraction A (100 mg.) was incubated with hyaluronidase (Benger's Hyalase, 1500 i.u.) in a phosphate–citrate buffer (0·15M-phosphate, pH 5·0, 25 ml.) for 90 hr. at 25°. After dialysis of the incubation mixture, 79 mg. of non-dialysable material was precipitated after addition of acetone (5 vol.) and solid NaCl (to 0·2M.) (Analysis: sialic acid, 4·6; hexuronic acid, 8·8%.) Further incubation of this product with hyaluronidase (4500 i.u., 72 hr., 25°), followed by dialysis and precipitation of the incubation mixture as described above, yielded 53 mg. (Analysis: sialic acid, 4·9; hexuronic acid, 7·4%.) Both the yields and analysis were obtained after correction for the hyaluronidase present. The activity of the hyaluronidase was checked as follows. Incubation of chondroitin sulphate (20 mg.) with hyaluronidase (1800 i.u., added at 2-day intervals in three equal amounts over a 7-day period) resulted in the diffusion of 0·13 mg. of hexuronic acid after dialysis of the incubation mixture against water (10 ml., 24 hr.).

Treatment with 5-aminoacridine hydrochloride

5-Aminoacridine–HCl, which forms insoluble salts with organic sulphates (Dodgson, Rose & Spencer, 1954), has been used by Muir (1958) to decrease the protein content of a chondroitin sulphate–protein complex obtained under neutral conditions from cartilage. This procedure was used to determine if a chondroitin sulphate preparation free of sialic acid could be precipitated from fraction A.

Fraction A (50 mg.) was dissolved in water (17 ml.) and the solution made acid to methyl orange with acetic acid. After centrifuging off and discarding the small amount of insoluble material, a sat. aq. solution of 5-aminoacridine–HCl was added slowly until no more precipitate had formed. After standing overnight at 4° the precipitate was centrifuged down, washed three times with water and suspended in 2·5% (w/v) sodium formate (8 ml.). The suspension was shaken with Zeo-Karb 225 (Na^+) (The Permutit Co. Ltd.) until the supernatant fluid showed no blue fluorescence. The resin was removed and washed four times with 3 ml. portions of water. The supernatant fluid and washings were combined, dialysed against distilled water and precipitation with the hydrochloride was repeated. The final product was precipitated from the dialysed solution by the addition of ethanol solution (4 vol.), followed by washing with ethanol and ether. The yield, after drying *in vacuo*, was 27 mg. (Analysis: sialic acid, 4·2; hexuronic acid, 4·2; protein, 27·0%.) The amino acid composition was similar to that of fraction A, except that no phosphoserine could be detected. A control experiment showed that orosomucoid (2 mg./ml.) and *N*-acetylneuraminic acid (1 mg./ml.) formed no precipitate under these conditions.

Treatment with alkali

Fraction A (40 mg.) was dissolved in NaOH (0·2N, 5 ml.) and incubated at 37° for 33 hr. To the resulting non-viscous, slightly yellow solution, acetic acid was added, to pH 5·4, and the solution was dialysed against water (10 ml.) for 24 hr.

Analysis of the diffusate showed that 44, 12 and 2% of the sialic acid, peptide material and hexuronic acid respectively had diffused out. The dialysate was then exhaustively dialysed against water and the barium salt of chondroitin sulphate was isolated from the dialysed solution by the method of Malawista & Schubert (1958) as follows. Barium chloride (50 mg.) was added to the dialysate, followed by ethanol to 20% (v/v). The precipitate (R_1) was collected, washed with ethanol and ether and dried *in vacuo*. This yielded 7·6 mg. of a light-brown material (Analysis: protein, 41; hexuronic acid, 5·7; sialic acid, 0%.) Ethanol was added to the supernatant fluid to 50% (v/v) and the precipitate (R_2) centrifuged down. The supernatant fluid (S) was retained. R_2 was redissolved in water (5 ml.), the reprecipitation with ethanol to 50% (v/v) in the presence of barium ions was repeated and the product washed with ethanol and ether. Ethanol (3 vol.) was added to the supernatant fluid (S) and the precipitate (R_3) washed with ethanol and ether. The amounts of R_2 and R_3 were too small to collect, and they were therefore dissolved in water (3 ml.) for purposes of analysis. The amounts of protein, sialic acid and hexuronic acid present in R_1, R_2 and R_3 are shown in Table 6.

Examination of chondroitin sulphate–protein complexes for the presence of sialic acid

The three chondroitin sulphate–protein complexes described under Methods and Materials were investigated. Portions (70 mg.) of each fraction were suspended in 0·01N-HCl (5 ml.) and the mixtures adjusted to pH 3 with 0·1N-HCl. The mixtures were then heated at 80° for 1 hr. Each hydrolysate was then dialysed against water (10 ml.) for 24 hr., followed by a second dialysis against a fresh 10 ml. portion of water for a further 24 hr. The diffusates (20 ml.) were combined, adjusted to pH 7 with 0·1N-NaOH and freeze-dried. The residues were dissolved in water (4 ml.) and 1 ml. was used for colorimetric estimation of sialic acid with both the diphenylamine and resorcinol reactions. The results are shown in Table 7. The absorption curves over the range 450–850 mμ of the chromogens formed after heating chondroitin sulphate A (pig trachea) and chondroitin sulphate A (human aorta) complexes, after the above-described treatment, with both the diphenylamine and resorcinol reagents, were identical with the chromogens produced with authentic *N*-acetylneuraminic acid.

Table 6. *Composition of fractions prepared from alkali-digested fraction A by fractional precipitation with ethanol*

Fraction A (40 mg.) was treated with NaOH (0·2N, 37°, 33 hr.). Fractional ethanol precipitation of the dialysed mixture was then carried out in the presence of Ba^{2+} ions. R_1, R_2 and R_3 were precipitated at ethanol concentrations of 20, 20–50 and 50–80% (v/v) respectively.

Fraction	Protein* (mg.)	Sialic acid (mg.)	Hexuronic acid (mg.)
R_1	3·1	0	0·43
R_2	0·3	0	1·13
R_3	0·6	0·5	0·11

* Method of Lowry *et al.* (1951)

DISCUSSION

Mechanical disintegration of cartilage at −196° produced a finely divided product, which, after warming to above 0°, dispersed readily in the solvents used for extraction. This procedure obviated the disadvantages attending high-speed homogenization of the tissue in large volumes of water. Extraction of the disintegrated cartilage at three different pH values (Table 1) showed, in agreement with results of other workers, that increasing amounts of material could be extracted as the pH was increased. Although alkali is particularly effective in releasing soluble cartilage constituents a certain amount of degradation undoubtedly occurs, as judged by the relatively low molecular weights of some chondroitin sulphate preparations (Mathews & Dorfman, 1953). On the other hand, less than 10% of the chondroitin sulphate in cartilage is extracted by neutral calcium chloride solutions (Mathews & Dorfman, 1953), and this material may not be entirely representative of the whole. In the bulk extraction of cartilage described in the present work a compromise was therefore reached whereby the pH was kept as low as possible, at the same time assuring a reasonably high yield of water-soluble constituents.

Table 7. *Sialic acid content of chondroitin sulphate–peptide complexes*

Methods used in isolating the complexes are given under Methods and Materials.

Complex	Source	Sialic acid (%) Diphenylamine reaction	Sialic acid (%) Resorcinol reaction
Chondroitin sulphate A	Pig tracheal cartilage	0·55	0·44
Chondroitin sulphate A	Human aorta	0·29	0·17
Chondroitin sulphate B	Human aorta	0	0

Under the mildly alkaline conditions used, the total yield from 100 g. of wet cartilage was 3·7 g. and analysis (Table 2) showed that 85% of the material could be accounted for on the basis of protein, sulphate, sialic acid, hexosamine and total non-nitrogenous hexose. The results for sulphur analysis were expressed in terms of sulphate since no sulphur-containing amino acids could be detected in any of the three fractions. The small amount of hydroxyproline in fraction A indicated that collagen was not a major protein constituent of this fraction. The most marked chemical difference between these fractions was the absence of appreciable amounts of hexuronic acid in fraction B_2, indicating the absence of chondroitin sulphates. Glegg *et al.* (1954) and Glegg & Eidinger (1955) have reported similar chondroitin sulphate-free fractions from connective tissue and demineralized bone from cattle.

The diphenylamine reaction for estimating sialic acid has been investigated by several workers (Anderson & Maclagan, 1955; Courts, 1959), and the violet chromogen produced, with a typical absorption maximum at 525 mμ, is thought to be specific for both the free and bound compound. The three fractions obtained from cartilage gave a similar chromogen with a strong absorption at 525 mμ (Fig. 1), which could not have been due to the presence in these fractions of non-nitrogenous hexoses or hexuronic acid, both of which gave small absorption maxima at 625 mμ. The absorption maxima (620–630 mμ) shown by a commercial sample of chondroitin sulphate and a chondroitin sulphate A–peptide complex isolated from pig tracheal cartilage (Muir, 1958) (Fig. 2) were no doubt due to the large amount of hexuronic acid present in these fractions. The second maxima (625–675 mμ) shown by the three cartilage fractions (Fig. 1) were probably due to their content of non-nitrogenous hexose and hexuronic acid.

Additional confirmation for the presence of sialic acid in fraction A was obtained after mild acid hydrolysis (Fig. 3). These results were similar to those obtained after mild acid hydrolysis of other sialic acid-containing fractions, particularly human-serum orosomucoid (Winzler, 1958), human-plasma α_1-acid glycoprotein (Schmid, Bencze, Nussbaumer & Wehrmüller, 1959), erythropoietin (Rambach, Shaw, Cooper & Alt, 1958) and bovine salivary glycoprotein (Gottschalk & Graham, 1959), where complete cleavage of sialic acid has been found associated with only traces of hexoses, more drastic hydrolysis being needed for the complete cleavage of hexoses and for the liberation of hexosamines. The chromogen formed on heating the non-dialysable components of hydrolysed fraction A with the diphenylamine reagent showed no significant maximum at 525 mμ (Fig. 3), indicating the complete removal of sialic acid. The modified non-dialysable product was partially denatured, as judged by solubility in water, and produced only a slight trace of humin material when heated with 5N-hydrochloric acid. Both free and bound sialic acid are known to produce considerable amounts of humin material when heated with strong mineral acid.

In the resorcinol reaction, although the chromogens produced with hexoses and hexuronic acid do not show absorption maxima at the wavelength (575 mμ) characteristic for sialic acid they absorb strongly enough at 450–500 mμ to interfere seriously with the colorimetric estimation of sialic acid (Fig. 4). However, mild acid hydrolysis of fraction A, which does not liberate dialysable hexuronic acid, and only small amounts of hexoses (Table 3), does liberate dialysable material which forms a chromogen identical to that formed by authentic *N*-acetylneuraminic acid. Hydrolysis of the cartilage fractions under more acid conditions, followed by removal of interfering substances by the method developed by Svennerholm (1958), also confirms the presence of sialic acid with the resorcinol (Fig. 4) and the diphenylamine reactions.

The amount of sialic acid thus found present in human cartilage would indicate that sialic acid-containing components form a substantial part of the polysaccharide fraction of the ground substance, increasing amounts of which could be extracted as the pH of the extracting medium was raised (Table 1).

Treatment of fraction A by mild chemical and enzymic procedures which have been used to decrease the nitrogen content of mucopolysaccharide preparations failed to separate completely the protein and sialic acid-containing components from the mucopolysaccharides. Moreover, the distribution of these components in the various fractions obtained by these methods was often at variance with the results obtained with purified chondroitin sulphate preparations from cartilage and glycoprotein preparations from human urine and serum.

Thus deproteinization attempts with phosphotungstic acid, followed by trypsin-treatment of the soluble fraction (R_2, Table 4), resulted in a product still containing protein and sialic acid-containing components. These results are similar to those of Webber & Bayley (1956), who found it impossible, with the same method, to obtain protein-free chondroitin sulphate from bovine nasal-septa extracts. Two other deproteinization treatments of fraction A, with chloroform–amyl alcohol and Lloyd's reagent, similarly failed to remove peptide material and sialic acid-containing components from the mucopolysaccharides.

Anomalous behaviour was also noticed when

fraction A was subjected to the benzoic acid-adsorption method, which has been used to obtain urinary glycoproteins virtually free of mucopolysaccharides (Anderson & Maclagan, 1955). It was found that adsorption on to benzoic acid of purified chondroitin sulphate was negligible.

Only when fraction A was treated with relatively strong alkali was it found possible to remove all the sialic acid and most of the peptide material from the mucopolysaccharide constituents. Such treatment did not result in the liberation of dialysable hexuronide. These results indicate, in agreement with other workers, that protein-free chondroitin sulphate preparations can only be obtained if the cartilage extract is first subjected to treatment with relatively strong alkali. It must also be concluded that similar difficulties exist in the removal of the sialic acid-containing components.

Three explanations are possible for this close relationship between sialic acid and hexuronic acid in cartilage extracts. Firstly, the protein moiety of the mucopolysaccharide–protein complex may be a sialoprotein of a type similar to those present in urine and serum. Partridge & Davis (1958) have detected glucosamine in the protein moiety of a mucopolysaccharide–protein complex extracted under mild alkaline conditions from bovine nasal septum. They concluded that the low nitrogen and amino acid content of this moiety indicated that only two-thirds was polypeptide material, and that the remainder must therefore contain components other than amino acids. Secondly, sialic acid may be involved in the linkage of the mucopolysaccharide molecule to the protein component, since theoretically sialic acid can enter into combination with sugars and amino acids through both glycosidic and peptide bonds. Lastly, sialic acid may be part of a glycoprotein, or mixture of glycoproteins, quite unrelated to any mucopolysaccharide–protein complex. In this case, failure to separate the glycoproteins from mucopolysaccharide material would merely be due to non-specific coprecipitation.

The presence of sialic acid in chondroitin sulphate A–peptide complexes isolated from pig trachea and human aorta by rigorous methods designed specifically to remove contaminating material would seem to indicate that sialic acid is, in fact, an integral part of these molecules. Further, the absence of sialic acid in the chondroitin sulphate B–peptide complex isolated from human aorta by similar methods indicates that contaminating sialic acid-containing components associated with this fraction have been effectively removed. The presence of sialic acid in the two preparations of chondroitin sulphate A and its absence in the chondroitin sulphate B preparation may reflect some fundamental differences in the nature of either the mucopolysaccharide–peptide links or in the peptide fragments themselves.

Both these chondroitin sulphate A complexes were isolated below pH 7·3. When subjected to pH values above 8·5, followed by ultrafiltration, amino acid-free polysaccharides can be isolated, the amino acid-containing portion appearing in the ultrafiltrate (H. Muir, personal communication). It is unlikely therefore that fraction A, isolated from cartilage at pH 10·6, contains the same complexes. However, the presence of more alkali-stable sialic acid-containing chondroitin sulphate complexes, which can only be isolated under more alkaline conditions, cannot be excluded.

SUMMARY

1. The specificities of the diphenylamine and resorcinol reactions for sialic acid in the presence of protein and acid mucopolysaccharide components were investigated, and the violet and blue chromogens absorbing strongly at 525 mμ and 575 mμ respectively were found specific for this compound.

2. Disintegration of human cartilage was carried out mechanically at $-196°$ and the amounts of water-soluble material extractable at three pH values were determined. Sialic acid-containing components were present in these fractions, increasing amounts of which could be extracted from cartilage as the pH was increased.

3. Three fractions were extracted from the disintegrated cartilage: a major fraction at pH 10·6 and two smaller fractions at pH 11·6. Analysis for protein, sulphate, sialic acid, hexosamine and total non-nitrogenous hexoses accounted for 85% by weight of the total solids. No sulphur-containing amino acids were detected.

4. Sialic acid was detected in certain mucopolysaccharide–peptide complexes.

5. The difficulties attending the separation of protein from acid mucopolysaccharide preparations were found to apply equally to the removal of the sialic acid-containing components. The significance of this is discussed.

I wish to thank Dr C. H. Lack for his interest and encouragement in this work and also for obtaining the specimens of cartilage. I am grateful to Dr J. T. Scales for permission to use the disintegrator mill. Part of this research was carried out with equipment purchased from a grant from the Dowager Countess Eleanor Peel Medical Research Trust, and also from a grant from the University of London to Dr C. H. Lack, in whose Department this work was done.

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The Preparation and Properties of Cystine Esterase

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Hooper & Jessup (1959) showed that placentae contain separate enzyme systems destroying oxytocin and vasopressin. During the purification of the enzymes, it seemed desirable to replace the biological estimation of oxytocinase by a chemical method using synthetic substrates. Certain properties of oxytocinase were revealed by inhibitors (Hooper, 1959), and it was concluded that the enzyme is a peptidase with esterase activity. Attempts have been made to find a chemical method of assay based on the presumed esterase activity of oxytocinase. A variety of esters was incubated with crude preparations of the enzyme and an additional enzyme which hydrolysed esters of cystine and of *S*-benzylcysteine, which has been called cystine esterase, was found. This report describes some of its specificities and its partial purification.

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METHODS

Estimation of amino acids. Amino acid ester hydrochlorides were incubated with enzyme as described below. Samples equivalent to approx. 100 μg. of original substrate were chromatographed on Whatman no. 1 paper with butan-1-ol–acetic acid–water (5:1:4, by vol.). After spraying with ninhydrin solution (0·1% in butan-1-ol–2% acetic acid) the colour was developed at room temperature for 1 hr., followed by heating at 80° for 5 min. Spots produced by amino acids were cut out, the colour was eluted with 3 ml. of propan-1-ol–water (1:1, v/v), and the extinction at 580 mμ measured. Determinations were done

in duplicate or triplicate, and control samples of ester and known amounts of amino acids were chromatographed concurrently with the products of enzyme digestion.

Estimation of enzyme activity. The standard conditions used in most experiments were as follows: enzyme preparations were incubated at 37° with *S*-benzyl-L-cysteine ethyl ester hydrochloride (5·5 mg./ml.; 0·02M) in 0·1M-phosphate buffer, pH 7·7. Samples (20 μl.) were withdrawn at intervals and the amount of *S*-benzyl-L-cysteine formed was estimated as described above.

Preparation of amino acid ester hydrochlorides. The ethyl ester hydrochlorides of the following amino acids were made according to the Fischer method: DL-alanine, L-arginine, L-aspartic acid, L-cystine, *S*-benzyl-L-cysteine, glycine, L-glutamic acid, L-histidine, DL-leucine, L-lysine, DL-methionine, DL-phenylalanine, DL-serine, DL-threonine and L-tyrosine. *S*-Benzyl-DL-cysteine was prepared by treating the L isomer with acetic anhydride (Wood & du Vigneaud, 1939) and was esterified by the above-described method.

Buffers. Phosphate buffers (NaH_2PO_4–Na_2HPO_4) were used to maintain hydrogen-ion concentrations between pH 5·5 and 8·5. Below pH 5·5 acetate buffers (sodium acetate–acetic acid) were employed, and above pH 8·5 borate buffers (sodium borate–boric acid) were used. All buffers were used at 0·1M-concentrations unless stated otherwise.

RESULTS

Partial purification of enzyme. The enzyme was obtained from human placentae; all operations were done near +2°. Tissue was ground with sand and 0·25M-sucrose solution (1 ml./g. wet wt.). The supernatant obtained after centrifuging the homogenate at 30 000 *g* for 30 min. was made 45% saturated with respect to ammonium sulphate by addition of 0·8 vol. of saturated ammonium sulphate solution. After the solution was stirred for 30 min. the precipitate was sedimented and discarded. The supernatant was then made approx. 65% saturated by addition of 1 vol. of saturated ammonium sulphate solution. The precipitate was collected and the supernatant discarded. The precipitate was dissolved in the minimum volume of 0·01M-phosphate buffer, pH 8·0, and dialysed against similar buffer to remove ammonium sulphate. The solution, which had been clarified by centrifuging, was chromatographed on an hydroxyapatite column (Tiselius, Hjertén & Levin, 1956) equilibrated with 0·01M-phosphate buffer, pH 8·0. Proteins were eluted by a stepwise increase in molarity of phosphate buffers, pH 8·0. The molarity of the buffers used and the elution pattern of protein measured at 280 mμ are shown in Fig. 1. The eluate containing the major peak of enzyme activity was dialysed against water and freeze-dried. The powder formed a pale-pink solution when dissolved in water.

pH *of optimum enzyme activity.* The variation of enzyme activity with hydrogen-ion concentration is shown in Fig. 2. The non-enzymic hydrolysis of the substrate at the different pH values selected was determined by incubation in the absence of enzyme; the corrections obtained have been applied to Fig. 2. The activity curve shows an optimum pH of 7·7–7·8.

Reaction with inhibitors. The crude preparation obtained by ammonium sulphate precipitation possessed both cystine-esterase and oxytocinase activities. The preparation was treated for 15 min. at pH 7·7 with enzyme inhibitors over a range of concentrations before addition of *S*-benzyl-L-cysteine ethyl ester hydrochloride or oxytocin (Pitocin; Parke, Davis and Co. Ltd.). Residual oxytocin was assayed on the isolated uterus of the rat and converted into oxytocinase units by means

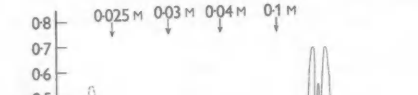

Fig. 1. Chromatography of impure enzyme on hydroxyapatite column (25 cm. × 2 cm.); 5 ml. fractions were taken. Continuous line is protein concentration measured at 280 mμ. Fractions were incubated with *S*-benzyl-L-cysteine ethyl ester hydrochloride for 4 hr. Amino acid formed was developed with ninhydrin and extinction at 580 mμ was measured (broken line). Molarity of eluting buffers was increased where indicated by arrows.

Fig. 2. Activity of enzyme as a function of hydrogen-ion concentration. Enzyme was incubated with substrate (0·02M) for 4 hr. at the pH values indicated; buffers were 0·1M. Liberated *S*-benzyl-L-cysteine was developed with ninhydrin as described in the Methods section.

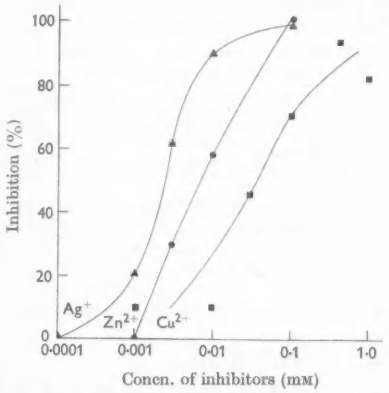

Fig. 3. Inhibition curves of oxytocinase treated with Cu^{2+}, Ag^{+} or Zn^{2+} ions. Enzyme was treated with inhibitors for 15 min. at pH 7·7 before addition of oxytocin. Reaction mixture was then incubated for 30 min. at 37°. Residual oxytocin was assayed on isolated rat uterus and converted into enzyme units as described by Hooper (1959). ■, Cu^{2+} (1 mM); ▲, Ag^{+} (0·1 mM); ●, Zn^{2+} (0·1 mM).

of a reference graph (Hooper & Jessup, 1959). Cystine-esterase activity was also measured. Fig. 3 shows the effect of three inhibitors on oxytocinase; Cu^{2+} (1 mM), Ag^{+} (0·1 mM) and Zn^{2+} (0·1 mM) ions caused virtually complete inhibition of oxytocinase. Cystine esterase was unaffected by these three metal ions at 1 mM-concentrations.

Specificity. After incubation for 18 hr., the ethyl ester hydrochlorides of the following amino acids were not hydrolysed to any detectable extent by the enzyme: DL-alanine, L-arginine, L-aspartic acid, glycine, L-glutamine, L-histidine, DL-leucine, L-lysine, DL-methionine, DL-serine, DL-threonine and L-tyrosine. Under the same conditions, *S*-benzyl-L-cysteine ethyl ester hydrochloride was converted completely into a substance chromatographically indistinguishable from the parent amino acid. L-Cystine diethyl ester hydrochloride gave a number of spots, the predominant one corresponding to L-cystine.

DISCUSSION

The estimation of amino acids as described here is not the most sensitive of methods. Estimation by development with Moore & Stein (1948) reagent after elution of amino acids from chromatograms was approximately three times more sensitive. When determinations were made on products of enzyme digestion, however, blank measurements with the Moore & Stein reagent were high and variable. The less-sensitive but more-reproducible method of elution of the developed colour was preferred.

S-Benzyl-L-cysteine ethyl ester hydrochloride was chosen as substrate for several reasons. First, the production of several spots by enzymic digestion of L-cystine diethyl ester hydrochloride suggested that disulphide interchange, analogous to that described by Ryle & Sanger (1955), occurred between L-cystine and the ester. Secondly, *S*-benzyl-L-cysteine ethyl ester hydrochloride is relatively easy to prepare in a pure form whereas L-cystine could not be obtained free from L-tyrosine, and the corresponding ester was not chromatographically pure. *S*-Benzyl-L-cysteine ethyl ester hydrochloride proved to be relatively stable under the conditions of incubation and corrections for non-enzymic hydrolysis were small.

In addition to undergoing hydrolysis by homogenate of human placenta, *S*-benzyl-L-cysteine ethyl ester hydrochloride was rapidly hydrolysed by homogenates of rat liver, kidney, heart, lung and spleen, and to a lesser extent by those of uterus and brain. Esterases are widely distributed (Ammon & Jaarma, 1951), and many do not appear to have high substrate specificities. It is possible that several enzymes may be responsible for the hydrolysis of *S*-benzyl-L-cysteine ethyl ester hydrochloride by tissue homogenates. During the preliminary stages of purification, advantage was taken of the fact that cystine esterase was precipitated along with oxytocinase during the ammonium sulphate-precipitation step. It is not possible therefore to give an indication of the purification achieved, since in the early purification steps the enzyme was not estimated by hydrolysis of the ester.

Cystine esterase is not identical with oxytocinase. First, with an impure preparation containing both activities, oxytocinase was inhibited by concentrations of metals ineffective on cystine esterase. Secondly, oxytocinase is more heat-labile than is cystine esterase, the latter remaining partially active when heated at 60° for 5 min. whereas oxytocinase activity was not detectable after such treatment. Also, when chromatographed on a hydroxyapatite column the peak of cystine-esterase activity did not coincide with any one of the peaks of oxytocinase activity.

Cystine esterase shows an interesting specificity. In the configuration $H_2N{\cdot}CH(CH_2{\cdot}S{\cdot}R){\cdot}CO_2R'$ the presence of the group $R{\cdot}S{\cdot}CH_2$- appears to be necessary for enzymic fission of the hydrolysable bond at R′. The substrate specificity of the enzyme has not yet been investigated in any great detail. Preliminary results suggest that the nature of R′ is not highly specific since methyl, ethyl and benzyl esters are hydrolysed. In experiments with

S-benzyl-DL-cysteine ethyl ester hydrochloride as substrate, the D isomer did not act as a competitive inhibitor. The specificity of cystine esterase is different from that of chymotrypsin and trypsin. Chymotrypsin requires the presence of an aromatic group for activity, and the hydrolysis of L-tyrosine ethyl ester may be used as a method of assay (Balls & Jansen, 1952). Trypsin requires the presence of basic groups and rapidly hydrolyses L-lysine ethyl ester (Dixon & Webb, 1958). In the conditions described earlier, cystine esterase did not hydrolyse either of these esters to any detectable extent.

SUMMARY

1. An enzyme occurring in human placenta hydrolyses esters of cystine and *S*-benzylcysteine more rapidly than it does those of other amino acids. The enzyme has been partially purified by ammonium sulphate precipitation and chromatography on a calcium phosphate column.

The skilled technical assistance of Mrs M. Gordon and Mr J. Caird is gratefully acknowledged.

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Studies in the Biochemistry of Micro-organisms

108. METABOLITES OF *ALTERNARIA TENUIS* AUCT.: THE BIOSYNTHESIS OF TENUAZONIC ACID*

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Alternaria tenuis Auct., when grown in surface culture on Czapek–Dox medium, has been shown to produce several new metabolites. From the mycelium, Raistrick, Stickings & Thomas (1953) obtained alternariol and alternariol methyl ether, and showed the structure of alternariol to be 3:4′:5-trihydroxy-6′-methyldibenzo-α-pyrone. From the culture filtrates of various strains, Rosett, Sankhala, Stickings, Taylor & Thomas (1957) isolated several new substances of unknown structure. One of these, tenuazonic acid, was later shown by Stickings (1959) to be 3-acetyl-5-*sec.*-butyltetramic acid (I). Thomas (1959, 1961) has recently shown by tracer studies that the carbon skeleton of alternariol is derived from acetate, and has pointed out that it could be obtained by head-to-tail condensation of acetate units, with no necessity for oxidation or reduction steps. Stickings (1959) suggested that tenuazonic acid might be synthesized in the fungus from one molecule of L-isoleucine and two molecules of acetate, also without any oxidation or reduction steps, and we now report evidence which supports this proposal. A preliminary account of part of this work has been given (Stickings & Townsend, 1960).

A strain of *A. tenuis*, catalogue no. 628, was chosen for this investigation, since it had already been shown (Rosett *et al.* 1957) to give a good yield of tenuazonic acid but none of the other products. In order to decide the most suitable time for addition of tracer acetate, the growth of *A. tenuis* was studied over a period of 6 weeks. It was shown that, in undisturbed surface culture, the concentration of tenuazonic acid increased rapidly from a low value on the fifth day to a maximum on the thirteenth to the fourteenth days. Other aspects of the growth and production curves are discussed later.

Sodium [1-^{14}C]acetate was therefore added on the fifth day to growing cultures, and these were

* Part 107: Birkinshaw & Samant (1960).

harvested on the thirteenth day. The tenuazonic acid isolated was found to contain 4·5% of the added radioactivity, and was therefore degraded stepwise to ascertain the activity associated with each of the ten carbon atoms of the molecule. The degradation scheme is shown in Fig. 1: the carbon atoms are labelled arbitrarily to facilitate discussion. The acid hydrolysis product, 3-amino-4-methylhexan-2-one, was treated in the reaction solution with alkaline iodine, when iodoform was produced in good yield; the isoleucine formed was not isolated, but its presence was shown by paper chromatography and conversion in good yield into 2-methylbutanal on boiling with aqueous nin-

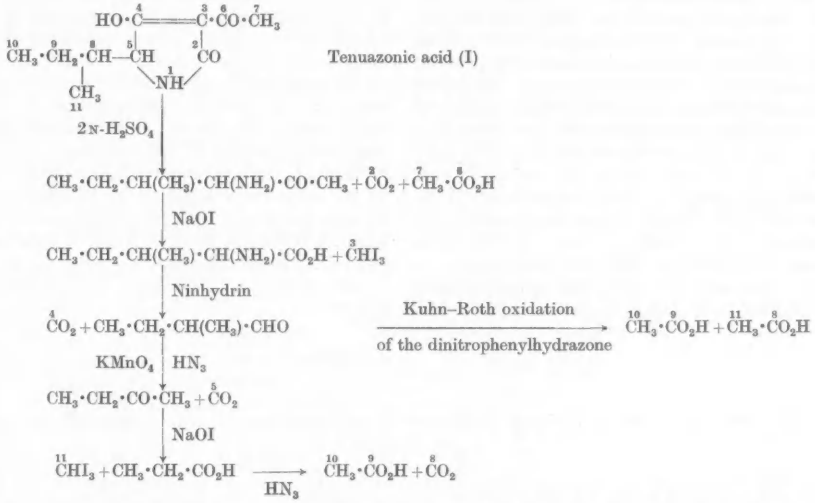


Fig. 1. Degradation scheme for tenuazonic acid. The numbering of the carbon atoms in the side chains is arbitrary. Acetic acid was degraded by the Schmidt reaction, or by the method of Popják (1955), to give each carbon atom as CO_2. Iodoform was also oxidized to CO_2.

Table 1. *Radioactivity of degradation products of tenuazonic acid*

The tenuazonic acid was produced by growing *A. tenuis* on Czapek–Dox medium with the addition of tracer sodium [1-^{14}C]acetate. Series I and II refer to two different lots of tenuazonic acid produced in this way. The degradation scheme and numbering are shown in Fig. 1.

		Series I			Series II		
Degradation product	Carbon atoms derived from tenuazonic acid	Activity of degradation product (μc/mole)	Activity of carbon atoms of tenuazonic acid (μc/mole of tenuazonic acid)	Fraction of total activity of tenuazonic acid (%)	Activity of degradation product (μc/mole)	Activity of carbon atoms of tenuazonic acid (μc/mole of tenuazonic acid)	Fraction of total activity of tenuazonic acid (%)
Barium carbonate	C-2	410	410	45·9	1162	1162	46·9
Barium carbonate	C-6	428	428	48·0	1197	1197	48·3
Barium carbonate	C-4	15·9	15·9	1·8	51·9	51·9	2·1
2-Methylbutanal 2:4-dinitrophenylhydrazone	C-5, -8, -9, -10 and -11	19·9	19·9	2·2	52·7	52·7	2·1
Barium carbonate	C-10, C-11	—	—	—	25·6	51·2	2·1
Barium carbonate	C-10	—	—	—	52·7	52·7	2·1
Barium carbonate	C-3, -5, -7, -8, -9 and -11 (all separately)	0	0	0	0	0	0
Total	C-2 to C-11	—	873·8	97·9	—	2463	99·4
Copper tenuazonate	C-2 to C-11	1785	893	100·0	4953	2477	100·0

hydrin. The 2-methylbutanal was degraded by the Schmidt and iodoform reactions (Strassman, Thomas, Locke & Weinhouse, 1956) and the activities of C-10 and C-11 were confirmed by a Kuhn–Roth oxidation.

The results are shown in Table 1, which also includes the results of an earlier incomplete series of degradations on a different sample of tenuazonic acid. It will be seen that the two series agree very closely, and that the bulk of the radioactivity (94–95%) is concentrated at C-2 and C-6 as expected. The remaining activity is shared equally between C-4 and C-10, and the corresponding atoms in isoleucine were found by Strassman *et al.* (1956) to be active when *Torulopsis utilis* was grown on a medium containing [1-^{14}C]acetate. They found 47 and 46% of the activity in the carboxyl and terminal methyl carbon atoms respectively. These results were part of the evidence in support of the biosynthesis of isoleucine from acetaldehyde (derived from pyruvate) and α-oxobutyrate.

It therefore seems probable that tenuazonic acid is synthesized in *A. tenuis* from two molecules of acetate and one of isoleucine, the latter being biosynthesized by a path identical with or very similar to that by which it is formed in *Torulopsis utilis*. The source of the labelling in the isoleucine is thought to be α-oxobutyrate, derived from oxaloacetate. Acetate would be incorporated into oxaloacetate by means of the citric acid cycle, but net production of oxaloacetate can only arise from other processes, such as the carboxylation of pyruvate. Pyruvate would be unlabelled, so also would the carbon dioxide arising from it during acetate formation. Some labelled carbon dioxide would arise during the citric acid-cycle reactions, and this, together with the operation of the cycle itself, could give rise to the labelling of the carboxyl-carbon atoms of oxaloacetate. It is clear, however, that this oxaloacetate would be expected to be very much less heavily labelled than the acetate, and this is in accord with the 20:1 ratio of labelling between C-2 and C-6 on the one hand, and C-4 and C-10 on the other.

This tentative biosynthetic path is outlined in Fig. 2.

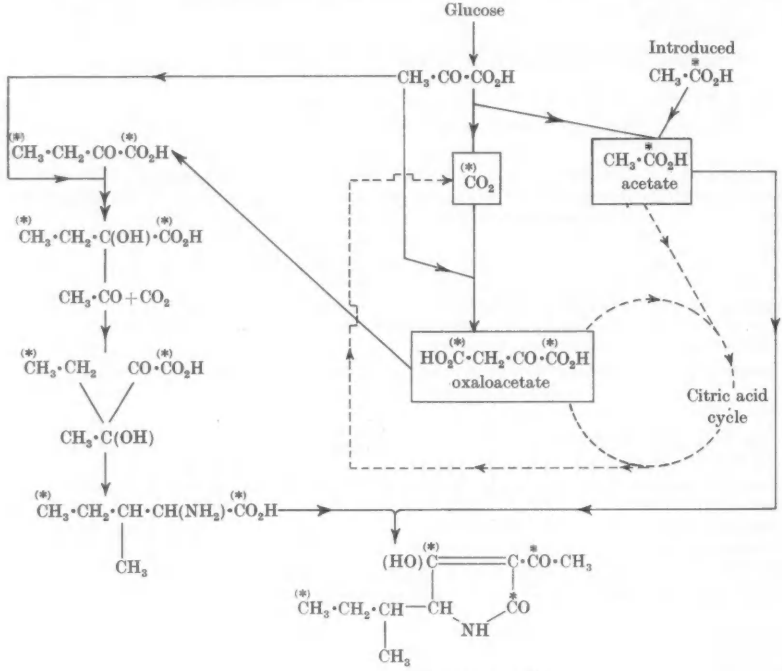
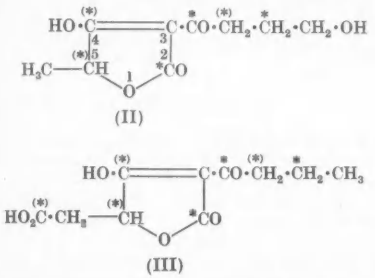


Fig. 2. Proposed biosynthetic scheme for tenuazonic acid, showing the labelling derived from [1-^{14}C]acetate. The intermediates in the biosynthesis of isoleucine are some of those proposed by Strassman *et al.* (1956). *, Major labelling; (*), minor labelling; ⟶, main biosynthetic path; - - - -→, source of minor labelling.

The present work affords no proof that the molecule of isoleucine is incorporated as a whole into tenuazonic acid; it may be that a precursor of isoleucine, rather than isoleucine itself, condenses with acetate or acetoacetate. If isoleucine is a direct precursor, the remaining stages could be via acetoacetylisoleucine, or deacetyltenuazonic acid (5-*sec.*-butyltetramic acid).

Lybing & Reio (1958) have made a similar study of the biosynthesis of carolic acid and carlosic acid by *Penicillium charlesii* G. Smith. These acids (II and III) are closely related structurally to tenuazonic acid, as are other substituted tetronic acids isolated from *P. charlesii*, *P. terrestre* Jensen, *P. cinerascens* Biourge and *P. viridicatum* Westling (Clutterbuck, Haworth, Raistrick, Smith & Stacey, 1934; Clutterbuck, Raistrick & Reuter, 1935; Birkinshaw & Raistrick, 1936; Bracken & Raistrick, 1947; Birkinshaw & Samant, 1960). Lybing & Reio (1958) found that radioactivity from [1-^{14}C]-acetate was incorporated mainly as shown in Fig. 3. The results are not as clear-cut as those we have found for tenuazonic acid, but the general picture is very similar. The degradation procedure was such as to make possible an equilibration in



(II)

(III)

Fig. 3. Labelling pattern in carolic acid (II) and carlosic acid (III) from *P. charlesii* grown on modified Czapek–Dox medium with the addition of tracer [1-^{14}C]acetate. *, Major labelling; (*), minor labelling [based on Lybing & Reio (1958)].

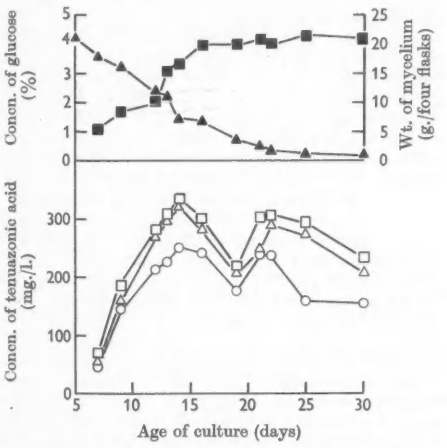


Fig. 4. Concentration of tenuazonic acid in filtrates from surface cultures of *A. tenuis*, on Czapek–Dox medium. Measurements were made on combined samples from four flasks. Calculated from: □, wt. of light petroleum extract; △, titration value of light petroleum extract; ○, wt. of copper tenuazonate isolated. ▲, Glucose (%), calculated from optical rotation; ■, total wt. of dried mycelium from four flasks.

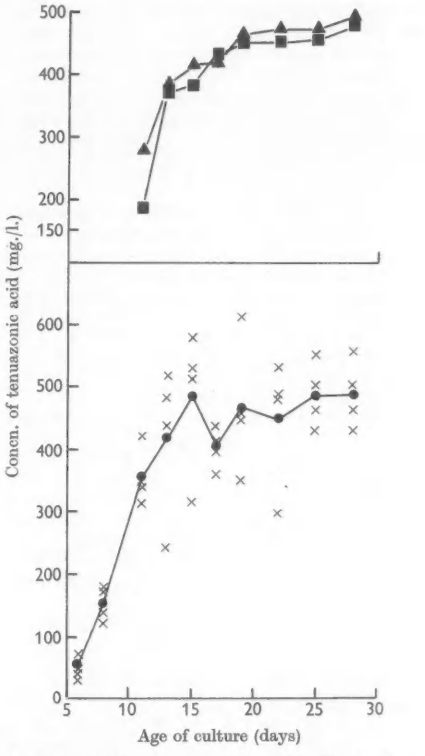


Fig. 5. Concentration of tenuazonic acid in filtrates from surface cultures of *A. tenuis*, calculated from *E* at 280 mμ. Four flasks were harvested on each day indicated: ×, separate values for the four flasks; ●, mean values. ■, Serial samples from one flask, taken with minimum disturbance of culture; ▲, serial samples from one flask, taken after mixing to ensure a representative sample. Curves for ■ and ▲ are displaced vertically to avoid confusion.

apparent activities at C-4 and C-5. Assuming that this originated mainly from C-4, the authors tentatively advance the theory that the tetronic acids arise by condensation of β-oxohexanoyl-coenzyme A and a C_4 compound of the citric acid cycle. The activity of C-4 and the carboxyl-carbon atom varied between one-quarter and one-sixth of that of the most active carbon atoms, marked with asterisks (a much higher fraction than the one-twentieth observed in tenuazonic acid). This could be due to greater citric acid-cycle action in *P. charlesii*, resulting in enhanced radioactivity in the carboxyl-carbon atoms of the C_4 acids.

Growth curves

The growth of the mould and production of tenuazonic acid were studied in five separate batches of culture flasks, by methods based on extraction procedures and u.v. absorption, which are described in detail in the Experimental section. Results for three batches are shown in Figs. 4–6. The u.v.-absorption figures gave higher values than those from the extraction procedure, but the shape of the curves was in general the same during the first 20 days.

Despite considerable variations between flasks (Fig. 5), there was in all five batches an initial rapid rise in mean concentration of tenuazonic acid to a maximum, followed by a drop to a minimum. Thereafter the behaviour was less uniform, but a fairly steady level was maintained. However, it was not possible to confirm this picture in single flasks, presumably due to the difficulty of obtaining a representative sample without disturbing the culture. The decrease in tenuazonic acid content after the initial rapid production period indicates that the metabolite can be utilized by the organism, at a time when the total glucose concentration is still quite high. On the other hand, there is no indication of substantial utilization of tenuazonic acid after most of the glucose is exhausted: thus in the first batch, the concentration of tenuazonic acid was practically unchanged from the twenty-seventh to the forty-fifth days.

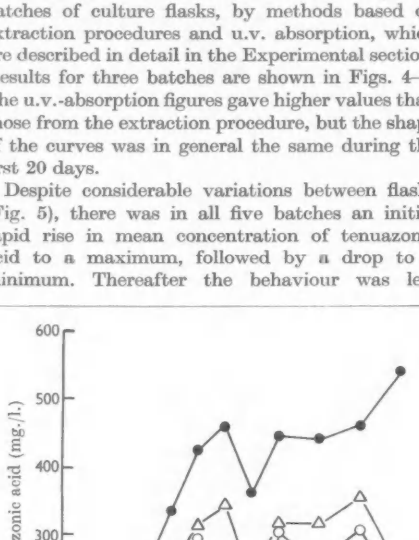


Fig. 6. Concentration of tenuazonic acid in filtrates from surface cultures of *A. tenuis*: comparison of methods of estimation. Four flasks were harvested on each day indicated. Calculated from: ●, mean value of *E* at 280 mμ; △, titration value of light petroleum extract; ○, wt. of copper tenuazonate isolated.

EXPERIMENTAL

Culture. The strain of *Alternaria tenuis* used, no. G.A. 628, has already been described (Rosett *et al.* 1957), together with details of cultural methods and of the isolation of tenuazonic acid. The mould was grown in the same way at 24° in surface culture in 1 l. conical flasks, each containing 350 ml. of Czapek–Dox medium.

Growth and production curves. (*a*) Extraction method. In order to determine the period of maximum tenuazonic acid production for the ^{14}C-incorporation experiments, a batch of flasks was sown and groups of four representative flasks were harvested at intervals. The following determinations were made on the combined filtrates on each occasion.

(1) Glucose concentration, determined approximately by optical rotation. (2) Mycelial weight (dry). (3) The culture filtrate was acidified to pH 2·0 and extracted by hand with light petroleum (b.p. 40–60°) until the extract no longer gave the typical orange–red ferric colour. The residue after evaporation of the solvent gave the weight of crude tenuazonic acid. (4) This residue was titrated with 0·1N-NaOH (to phenolphthalein); the titration value represents tenuazonic acid plus acidic impurities. (5) The titration solution was extracted with chloroform several times (this extract was rejected), then treated with slightly more than 1 equiv. of 0·1N-copper acetate and extracted again with chloroform until the extract no longer gave a ferric colour. The chloroform solution was evaporated and the residue dried at 100° (16 mm. Hg) and weighed. It contained all the tenuazonic acid as nearly pure copper salt.

The results were substantially confirmed when two further batches were followed by the same method.

Results for batch 3 are shown in Fig. 4.

(*b*) U.v. methods. The u.v.-absorption curve of the diluted culture filtrate was measured at various intervals. It was closely similar to that of pure tenuazonic acid, with interference from the medium below 250 mμ in the earlier stages of metabolism. The maximum at 279–280 mμ and the minimum at 255 mμ were observable at all times; from about the thirteenth day onwards, the second maximum at 240 mμ was also observed. The extinction at 280 mμ was therefore taken as a measure of the concentration of tenuazonic acid. Beer's law was checked by observing a series of dilutions of a sample of metabolism solution: dilutions of 1/21, 1/41, 1/61, 1/81, 1/101 gave the following values of *E* for the undiluted solution: 33·6, 33·5, 33·6, 32·8, 33·2.

The extinction (at 280 mμ) of appropriately diluted 1 ml. samples was measured in two further batches of culture flasks (nos. 4 and 5). Four representative flasks were again harvested at intervals. *E* (280 mμ) and glucose by optical rotation were measured on each flask. The values of *E* and their means, for batch 5, are shown in Fig. 5. There was no obvious correlation between values of *E* and glucose levels and these are not shown. With batch 4, portions (250 or 125 ml.) from each flask were combined and the extraction procedure described above was carried out to compare the two methods of estimation. The results are shown in Fig. 6.

In view of the higher figures obtained by the u.v. method, especially during the later stages of metabolism, the absorption curve of the extracted culture filtrate was measured. At 27 and 30 days, this gave general absorption over the range 230–290 mμ with a flat maximum at 260 mμ. The absorption of this residual material accounted for about 15% of the total absorption of the culture filtrate at 280 mμ.

Batches 4 and 5 also each included two flasks equipped with side arms and taps for sampling. In one of each pair the side arm extended to the middle of the solution, in the hope that a representative sample could be withdrawn without disturbing the cultures. The other flask was rotated gently to mix the solution before the sample was taken. Samples (1 ml.) were withdrawn from these flasks on the same days as the four-flask samples were taken, and *E* (280 mμ) was measured. The values for batch 5 are shown in Fig. 5; the curves for the single flasks in batch 4, and for a number of other single flasks, were very similar, and showed no distinct peaks.

The method of sowing used for batches 4 and 5 was modified to ensure sowing exclusively with spores, rather than with spores and mycelium. This resulted in a slower start in growth and probably accounts for the delayed maximum as compared with batches 1–3.

Radioactivity measurements. Substances were counted (usually for $3 \times 10\,000$ counts) at 'infinite thickness' on 1·5 cm.-diam. planchets with an end-window Geiger–Müller tube. Accuracy was ±3% for 10 000 counts. Background count (5–13 counts/min.) was subtracted in all cases, but no corrections were made for back-scattering. Three different tubes were used during the work, with standard counts of about 720, 910 and 1130 counts/min., with a 2·5 cm.-diam. disk of polymethylmethacrylate of nominal activity 1 μc/g. Results are accordingly calculated as μc/mole.

Incorporation of [1-^{14}C]acetate into tenuazonic acid. Sodium [1-^{14}C]acetate (50 μc; about 0·2 mg. in 1 ml. of sterile water) was added to each of two flasks of batch 2 on the sixth day after inoculation. The flasks were harvested and extracted on the thirteenth day as described above, and the crude copper salt (437 mg.) was recrystallized from aqueous methanol; $[\alpha]_{20}^{5461} - 122 \pm 1°$ (*c*, 0·2 in methanol); 4538 counts/min. ≡ 8980 μc/mole (yield 257 mg.; incorporation of tracer 4·5%).

In a repeat experiment, sodium [1-^{14}C]acetate (100 μc) was added to each of two flasks; yield, 471 mg. of purified copper salt, 10 440 μc/mole.

The copper salt was diluted with inactive material for the degradation experiments.

Complete degradation of tenuazonic acid

The scheme of degradation used is shown in Fig. 1, and the radioactivity measurements are given in Table 1. Each carbon atom was obtained as CO_2, which was converted into $BaCO_3$ for assay.

Hydrolysis. In a typical experiment, copper tenuazonate trihydrate (938 mg.) was converted into the free acid and hydrolysed as described by Stickings (1959), with 2N-H_2SO_4 (300 ml.) instead of HCl and omission of the Brady's reagent bubbler. The barium carbonate was filtered and dried (649 mg.). The acetic acid was degraded by the method of Popják (1955).

Reaction of 3-amino-4-methylhexan-2-one with alkaline hypoiodite and ninhydrin degradation of the resulting isoleucine. The hydrolysis reaction mixture was neutralized (NaOH), treated with $BaCl_2$ to remove SO_4^{2-} ions and evaporated under reduced pressure to a small volume. It was then treated with 0·1N-iodine solution (150 ml.) and 10N-NaOH (20 ml.) at 0°. Iodoform was immediately precipitated in good yield and was centrifuged and oxidized to CO_2 (Popják, 1955). The supernatant was acidified and the excess of iodine destroyed by titration with 0·1N-sodium thiosulphate. It was then neutralized, evaporated and made to a volume of 250 ml. with water. A drop of this solution was chromatographed on paper as described by Stickings (1959) and showed the presence of isoleucine. The solution was boiled with ninhydrin (1·22 g.) in a stream of N_2, and yielded 2-methylbutanal, isolated as its 2:4-dinitrophenylhydrazone, m.p. 136° (671 mg.). Further confirmation of the stability of isoleucine to alkaline hypoiodite was obtained by boiling an aqueous solution of the two substances for 1 hr., after which isoleucine could still be shown chromatographically to be present at about the same concentration.

Kuhn–Roth oxidation of 2-methylbutanal 2:4-dinitrophenylhydrazone. 2-Methylbutanal 2:4-dinitrophenylhydrazone (254 mg.) was heated for 4 hr. with a chromic acid mixture [CrO_3 (12 g.), $K_2Cr_2O_7$ (3 g.), water (30 ml.) and H_3PO_4 (20 ml.)]. The volatile acid was titrated (Found: 18·85 ml. of 0·1N-NaOH. Calc. for $2CH_3 \cdot CO_2H$: 19·08 ml.). The sodium acetate was degraded by the Schmidt reaction to CO_2 and methylamine, which was oxidized with permanganate to CO_2 (Phares, 1951).

Stepwise degradation of 2-methylbutanal 2:4-dinitrophenylhydrazone. The method followed closely that given by Strassman *et al.* (1956), with the conditions for the Schmidt reaction described by Phares (1951). Yields of $BaCO_3$ (from 245 mg. of 2-methylbutanal 2:4-dinitrophenylhydrazone) were: from C-5, 179 mg.; from C-11, 172 mg.; from C-8, 190 mg.; from C-9, 164 mg.; from C-10, 120 mg.

SUMMARY

1. A strain of *Alternaria tenuis*, growing on Czapek–Dox medium, has been shown to incorporate over 4% of the ^{14}C from added sodium [1-^{14}C]-acetate into tenuazonic acid.

2. Over 94% of the radioactivity is shared equally between the lactam and side-chain carbonyl groups; the remainder is shared equally between C-4 of the ring and the ω-carbon atom of the *sec.*-butyl side chain.

3. It is suggested that tenuazonic acid is synthesized from two molecules of acetate and a molecule of L-isoleucine.

4. The relationship of this pathway to the biosynthesis of fungal tetronic acids is discussed.

5. During growth of *A. tenuis* in undisturbed surface culture, the concentration of tenuazonic acid increases rapidly during the second week, then decreases to a minimum and rises again.

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Tissue-Specific and Species-Specific Esterases

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Although electrophoresis has been used extensively to study the protein components of biological fluids, few attempts have been made to investigate soluble proteins from solid tissues by this means, mainly because classical techniques give inadequate resolution of complex mixtures. Zone electrophoresis in starch gels (Smithies, 1955) permits sharper separation and greater resolution, and in the modification introduced by Hunter & Markert (1957) can also be used for specific proteins by applying methods derived from histochemistry. For instance, by applying the method of Nachlas & Seligman (1949) these authors demonstrated several bands with esterase activity in starch-gel-electrophoresis diagrams of aqueous extracts of mouse tissues.

Electrophoresis of serum proteins on starch gels has revealed differences between species (Latner & Zaki, 1957) and even among individuals of the same species (Ashton, 1957; Smithies, 1955, 1959). By other techniques Augustinsson (1958) has demonstrated that there are considerable differences in the serum esterases of different species.

In this investigation starch-gel electrophoresis was used to compare the tissue esterases of a variety of organs in several species, in order to investigate the amount of variation among proteins with the same enzyme activity. Typical patterns were found for each tissue, and their constancy was demonstrated by their persistence in mammalian cell strains, which had been cultivated *in vitro* for long periods.

MATERIALS AND METHODS

Tissues. Animals were killed by exsanguination after stunning or after anaesthetizing with ether. The organs were immediately removed and treated as described below. Human tissues were obtained from the post-mortem room, washed thoroughly with 0·15M-NaCl immediately after removal, and frozen at −10° until required. Cell strains were grown as monolayers on glass by standard techniques (Paul, 1959). They were washed *in situ* with 'balanced salt solution' (Hanks & Wallace, 1949) and harvested by scraping from the glass with a rubber 'policeman' before the preparation of extracts. Except for the strains described below, cells were derived from recently disaggregated tissues. Cell strains used were the mouse subcutaneous (fibroblast) strain L, clone 929 (Sanford, Earle & Likely, 1948), the human cervical carcinoma cell, strain HeLa (Gey, Coffman & Kubicek, 1952), the human epithelioma cell, strain HeP 1 (Toolan, 1954) and the human foetal liver (parenchymal) cell, strain HLM (Leslie, Fulton & Sinclair, 1956).

Preparation of extracts. The method of preparation of tissue extracts was found to affect the pattern obtained.

Methods tried included simple extraction with water, balanced salt solution and various buffer solutions and also extraction with the same solutions accompanied by ultrasonic treatment for various periods. Extraction with water or balanced salt solution (0·15 M) gave identical results, and the electrophoretic patterns obtained were not affected by ultrasonic treatment. However, when extraction was performed with 0·06 M-buffers [2-amino-2-hydroxymethyl-propane-1:3-diol (tris) or phosphate] at pH 7·3 or 8·3, different patterns were obtained (Fig. 1), and ultrasonic treatment led to the progressive disappearance of some bands and increased intensity of others on the electrophoresis pattern. The final procedures adopted were as follows.

Extracts from solid tissues. The tissue was first ground with 0·5 vol. of iso-osmotic salt solution or balanced salt solution in a Potter–Elvehjem homogenizer. When a fine uniform homogenate had been obtained it was placed in an ice bath and subjected to ultrasonic treatment for 15–20 min., with a Mullard ultrasonic drill (50 w, 20 kcyc./sec.).

Fig. 1. Comparison of the zymograms obtained from mouse-liver extracts prepared with (*A*) 0·06 M-phosphate buffer and (*B*) water. Relative intensities of bands are indicated by shading. (The phosphate buffer, pH 8·3, was a mixture of Na_2HPO_4 and NaH_2PO_4.)

Finally, it was centrifuged at 35 000 *g* for 2 hr. at 0°. The clear supernatant was used for electrophoresis.

Extracts from cultured cells. Cells were suspended in 0·5 vol. of balanced salt solution and subjected to ultrasonic treatment for 2–3 min. The suspension was centrifuged at 15 000 *g* for 2 hr. at 0°, and the clear supernatant used.

Electrophoresis. The apparatus and general method were essentially those of Smithies (1955). Single-electrode vessels were used at the anode and cathode, and simple carbon electrodes were found adequate. In earlier studies AnalaR soluble starch (British Drug Houses Ltd.) was used for making the gels, batches being tested until a suitable one was found. Starch Hydrolysed (Connaught Laboratories) was used in the later studies.

The discontinuous buffer system of Poulik (1957) was used. The gel was prepared in a buffer, pH 8·64, composed of 0·076 M-tris and 0·005 M-citric acid. While still fluid, the gel was covered with polythene film to prevent subsequent evaporation. The electrode vessels were filled with a borate buffer (0·3 M-boric acid–0·05 M-NaOH). The samples were introduced in filter paper as described by Smithies (1955). Small pieces of Whatman no. 3MM filter paper were cut to exactly the cross-section of the gel. After saturating them with the tissue extract they were inserted into slits in the gels, one-third of the way from the cathode.

Staining for proteins. The gels were split longitudinally and stained with Naphthalene Black 10B (G. T. Gurr Ltd., 136 New Kings Road, London, S.W. 6) as described by Smithies (1955).

Staining for esterases. Gomori's (1952) modification of Nachlas & Seligman's (1949) histochemical method for esterases was adapted as follows. A stock solution containing 1% (w/v) of α-naphthyl acetate in 50% (v/v) acetone was prepared. Immediately before use 0·8 ml. of this solution was added slowly with stirring to 50 ml. of water. Tetrazotized *o*-dianisidine (Fast Blue Salt B, G. T. Gurr Ltd.) (20–30 mg.) was then added to make the substrate mixture. The gels were first placed in 100 ml. of 0·25 M-phosphate buffer, pH 6·25. After 15 min., freshly made substrate mixture was added and the gels were incubated at 37° until adequate development was obtained. Background staining was removed by washing the gels with methanol–acetic acid–water (50:10:50, by vol.).

In those experiments designed to investigate the inhibitory effects of physostigmine salicylate and *NNN'N'*-tetra*iso*propylpyrophosphoramide the inhibitors were incorporated in the buffer used for making the gel. Subsequently, before staining the gels by the procedures outlined above, they were submerged for 30 min. in 0·25 M-phosphate buffer, pH 6·25, containing the inhibitor. The substrate mixture was added to this as described above.

Photography. The gel patterns were recorded by photography on Kodak microfile film, with standard exposure and development times determined experimentally. For photography of protein patterns a red filter was used and for photography of esterase patterns a blue filter.

Reproducibility of the method. An extract was prepared from the liver of a stock albino mouse and subjected to electrophoresis on four gels. The experiment was repeated on another occasion with the same extract. Identical electrophoresis patterns were obtained in all cases. Minor differences in the resolution of some bands were found with different batches of starch.

RESULTS

In the following account the term 'zymogram' (Hunter & Markert, 1957) is used to describe the pattern obtained when a tissue extract is separated by electrophoresis on a starch gel and this is subsequently stained to reveal the location of specific enzymes.

Relationship between distribution of esterases and protein. When two halves of the same gel were compared, one stained for protein and the other for esterase activity, no relationship between the bands of esterase activity and the protein bands could be recognized. For instance, the fast-moving esterase band observed in most tissues preceded and did not coincide with the albumin band. Similarly, the dense, slow-moving esterase band in human liver (Fig. 2) corresponded to an area with very little protein. On the other hand, areas of dense protein staining, e.g. serum albumin, frequently corresponded with areas devoid of esterase activity.

Esterases from different species. Zymograms prepared from the same organs of the mouse, guinea pig, rat, fowl and human were compared. Each species had its own distinct pattern (Fig. 2). Not only was there no evidence for the occurrence of the same protein in more than one species but the number of proteins with esterase activity varied

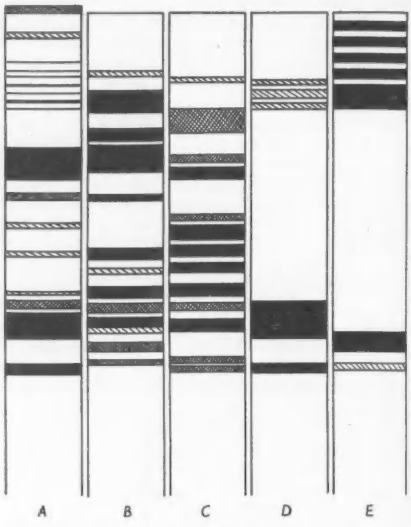

Fig. 2. Zymograms of liver extracts from different animals. In order to compare relative mobilities preparations were run in pairs in the same gels. Relative intensities of bands are indicated by shading. *A*, Mouse; *B*, rat; *C*, guinea pig; *D*, hen; *E*, human.

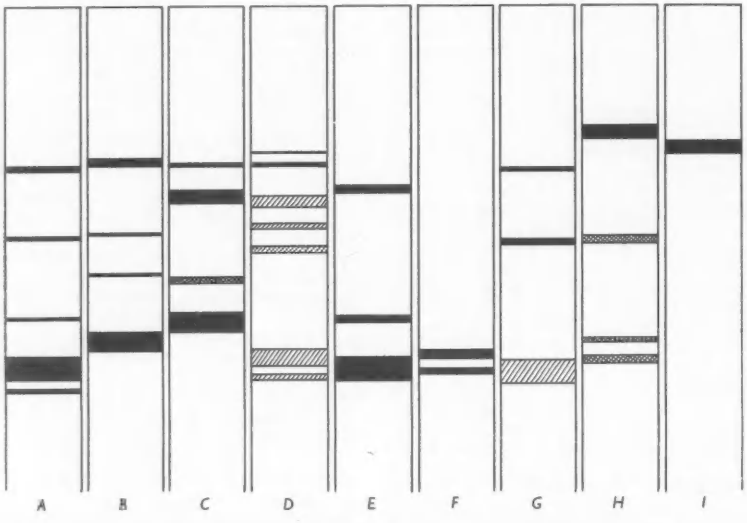

Fig. 3. Esterase zymograms from the serum of different animals. Relative intensities of bands are indicated by shading. *A*, Mouse; *B*, rat; *C*, guinea pig; *D*, human; *E*, rabbit; *F*, cow; *G*, lamb; *H*, chicken; *I*, horse.

very greatly from species to species. In the serum, too, as shown in Fig. 3 there were radical differences among the species examined. In all these experiments duplicate sections of the starch gels were stained for protein with Naphthalene Black 10B. Species differences were again apparent but were much less than in corresponding zymograms.

Tissue esterases in different individuals. Variations among individuals of the same species were investigated by preparing extracts from livers and kidneys of mice from a stock inbred strain maintained in the laboratory. In over 40 specimens the same sixteen bands were identified in liver preparations and the same twelve bands in kidney preparations, with only two exceptions. In one of these a single esterase was found to be missing from the liver zymogram, and in the other a single band was missing from liver and two bands (including the one missing from liver) were missing from kidney preparations. Fig. 4 demonstrates this latter case strikingly. The abnormal preparation was inserted in the same gel as a normal preparation and they were subjected to electrophoresis simultaneously.

Specimens from three adult humans and two human foetuses were also compared and identical patterns were always obtained from extracts of the same organs.

Esterases from different tissues. Fig. 5 represents typical zymograms obtained from extracts of liver, kidney, heart, muscle and serum of stock albino mice and illustrates that in agreement with Hunter & Markert (1957) quite different patterns are obtained from different organs in this animal. Liver exhibited a particularly complex pattern whereas at the other extreme skeletal muscle produced a simple one. Certain esterases were always found in all the mouse tissues investigated, providing a species characteristic.

Tissue from the guinea pig and rat show a similar variation from organ to organ.

Fig. 4. Comparison of liver extracts from a normal mouse and one in which an esterase was absent. The two preparations were inserted side by side in the gel.

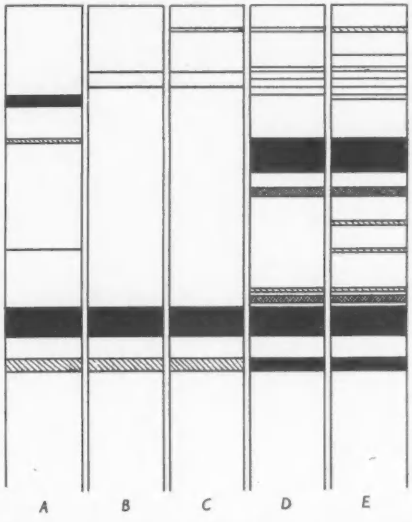

Fig. 5. Zymograms from extracts of different organs of the mouse. Relative intensities of bands are indicated by shading. *A*, Serum; *B*, skeletal muscle; *C*, heart; *D* kidney; *E*, liver.

In contrast, tissues from the human almost all exhibited the same pattern of seven bands. In one or two organs, notably liver, adrenal and thyroid, additional ones were observed but the differences were very minor compared with those observed in some other species (Fig. 6).

Since some variation was obtained in zymograms from different normal organs in the same animals, some tumour tissues were compared with corresponding normal tissues. In two of three human tumours, a hypernephroma and a carcinoma of the rectum, the zymograms were indistinguishable from those obtained from adrenal cortex and rectal mucosa respectively. In a third case, however, also a hypernephroma, a dense band was obtained in the zymogram which was not found in either kidney or adrenal tissue. The zymogram, in fact, most closely resembled that normally obtained from liver tissue.

Esterases in cultured cells. It has been suggested (Vandelli & Scaltriti, 1943) that esterases may be adaptive enzymes. However, the human pattern was retained in human cells which had been cultured *in vitro* for periods varying from a few days with kidney cells to many years with HeLa cells (Fig. 7). Further, some of the cells had been grown for at least 3 months in heterologous media containing calf serum, and cells originating from different species still gave different zymograms although they had been cultivated in the same media for many months. These findings suggest that the patterns obtained are not dependent on the immediate environmental conditions but more probably represent differences of a genetic nature.

Classification of esterases in zymograms. According to Augustinsson (1958) esterases can be distinguished on the basis of substrate specificities and inhibition by specific substances. Physostigmine specifically inhibits acetylcholinesterases (C esterases) at a concentration of 10^{-5} M, but this had no apparent effect on any of the esterases in zymograms from mouse liver although at 10^{-3} M three bands were inhibited. In human liver, treatment with 10^{-4} M-physostigmine salicylate produced a decrease in the intensity of all the bands whereas at 10^{-3} M all the bands were completely inhibited. Tetra*iso*propyl pyrophosphoramide specifically inhibits the ali-esterases (B esterases) as well as the C esterases. At a concentration of 10^{-4} M no inhibition of any of the bands on the zymograms from human or mouse material was obtained. At 10^{-3} M, partial inhibition of all the esterases of human liver and partial inhibition of one band in

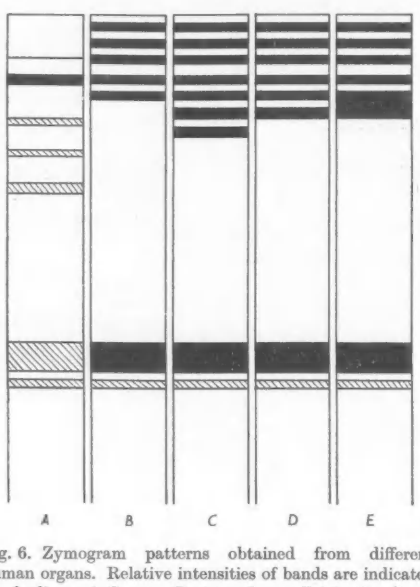

Fig. 6. Zymogram patterns obtained from different human organs. Relative intensities of bands are indicated by shading. *A*, Serum; *B*, stomach, small intestine, lung, thymus, adrenal, testis, spleen, heart; *C*, thyroid; *D*, kidney; *E*, liver.

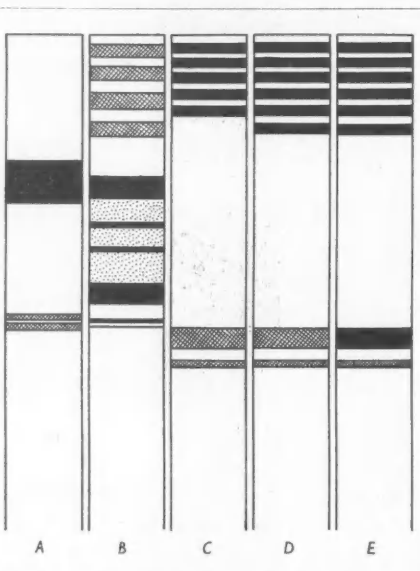

Fig. 7. Zymogram patterns from extracts of cultured cell strains compared with an extract of human kidney. Relative intensities of bands are indicated by shading. *A*, L 5178 Y (murine origin); *B*, strain L (murine origin); *C*, HeLa, HEP I, HLM, skin, heart (all of human origin); *D*, freshly cultured human-kidney cells; *E*, human kidney (not cultured).

mouse liver were obtained. It would seem therefore that the majority of the esterases revealed by this technique are non-specific aromatic esterases (A esterases).

DISCUSSION

In view of the high degree of species specificity shown by the patterns from different animals and their persistence during prolonged cultivation *in vitro* the use of these techniques for the identification of cell-strains is a practical application of immediate interest.

Of more fundamental interest is the observation that a very large number of bands with esterase activity can be demonstrated by starch-gel electrophoresis. Although these bands may be artifacts caused by the adsorption of esterases on to other proteins, this explanation seems unlikely, since there is no obvious correlation between zones of high esterase activity and zones of high protein concentration. The alternative explanation is that a very large number of proteins may display esterase activity even within the same organ, at least 16 in the mouse liver. Many examples of multiple forms of enzymes have been described in recent years. These include aldolase (Taylor, Green & Cori, 1948), lactic dehydrogenase (Meister, 1950; Nielands, 1952; Markert & Møller, 1959), lysozyme (Tallan & Stein, 1953), β-glucuronidase (Mills, Paul & Smith, 1953), ribonuclease (Hirs, Moore & Stein, 1953), triose phosphate dehydrogenase (Krebs, 1953), enolase (Malstrom, 1957), alkaline phosphatase (Schlamowitz, 1958), peroxidase (Markert & Møller, 1959), esterases (Hunter & Markert, 1957) and trypsin (Timasheff, Sturtevant & Bier, 1956). These multiple forms of enzymes have been described within the same species and when species differences are also considered it may reasonably be assumed that an extremely large number of proteins with the same kind of activity can exist.

Proteins possessing the same enzyme activity may be related in at least three ways. First, they may represent different degrees of polymerization of the same molecular species, as is known to occur in haemocyanin (Pedersen, 1949) and insulin (Rees & Singer, 1955). The fact that esterases in different tissues of the mouse occur in almost all possible combinations in electrophoresis diagrams argues against the existence of different polymers but, on the other hand, this would provide a reasonable explanation for the differences obtained when the tissues were extracted with buffer solutions as compared with water or saline.

The second possibility is that different molecular species may exist with the same enzyme specificity. Markert & Møller (1959) have proposed the term 'isozymes' for proteins related to each other in this way. The existence of isozymes is supported by evidence, such as that of Mills *et al.* (1953), that different proteins can be isolated with the same enzyme activity and also the findings that immunologically distinct lactic dehydrogenases (Niesselbaum & Bodansky, 1959) and phosphorylases (Henion & Sutherland, 1957) can be identified in different tissues of the same animal.

Finally, enzymes may possess different, but overlapping, specificities. Many esterases are, of course, known to fit into this category and it is well known that aromatic esters are hydrolysed by many enzymes whose specific function is quite different, e.g. chymotrypsin (Kaufman, Schwert & Neurath, 1948).

Since there is evidence that protein structure is under direct genetic control (Allison, 1959) the implication is that a considerable amount of genetic variation is compatible with performance of the same specific function. In order to reconcile this with the similarity of metabolic pathways at all levels of evolution, it is necessary to postulate a mechanism which permits very wide structural variations in a protein while at the same time maintaining function rigidly through an enormous number of generations. The most reasonable suggestion is that the structure of certain areas in an enzyme molecule are specific for enzyme function, whereas the rest of the molecule can vary widely without disturbing function significantly. There is some experimental support for this view. On the one hand, it has been shown that in different proteins with the same enzyme activity, certain areas of the molecule may be structurally similar and specific for enzyme function (Oosterbann, Jansz & Cohen, 1956; Koshland & Erwin, 1957; Oosterbann, Kunst, Van Rotterdam & Cohen, 1958; Porter, Rydon & Schofield, 1958), whereas, on the other hand, some enzyme proteins can be partially degraded without losing their functional activity (Pope, 1939; Perlmann, 1954; Richards, 1955; Harris & Li, 1955; Rogers & Kalnitsky, 1957; Hill & Smith, 1958).

It follows that there may be an infinite variety of protein molecules with the same functional activity. It is not surprising therefore that a great multiplicity of species-specific and even tissue-specific enzymes are revealed by starch-gel electrophoresis, an analytical tool with a resolving power which approaches that of immunological methods.

SUMMARY

1. The separation of tissue esterases by starch-gel electrophoresis is described.

2. Different species exhibit entirely different patterns by this technique and these are highly reproducible.

3. In some species considerable differences can be recognized between different tissues, although underlying species characteristics can always be recognized.

4. The patterns persist in cells which have been cultured *in vitro* for many years and are therefore genetically stable.

5. It is concluded that an enormous number of proteins with the same functional activity can exist and the significance of this observation is discussed.

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Anti-Inflammatory Drugs and Intermediary Metabolism

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Salicylate uncouples oxidative-phosphorylation reactions in respiring mitochondrial preparations (Brody, 1956), and many of its effects on the metabolism of animals and isolated tissues are explicable in terms of this action (Smith, 1959; Smith & Moses, 1960). However, the anti-inflammatory properties of salicylate must be mediated by mechanisms other than an uncoupling action, since 2:4-dinitrophenol, although a more powerful uncoupling reagent than salicylate, lacks anti-

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inflammatory effects (Adams & Cobb, 1958; Marks & Smith, 1960). The present study is concerned with the effects of four other anti-inflammatory drugs, butazolidin (3:5-dioxo-1:2-diphenyl-4-*n*-butylpyrazolidine), chloroquine diphosphate [7-chloro-4-(4-diethylamino-1-methylbutylamino)-quinoline diphosphate], cortisol (11β:17α:21-trihydroxypregn-4-ene-3:20-dione) and dexamethasone (16α-methyl-9α-fluoro-11β:17α:21-trihydroxypregna-1:4-diene-3:20-dione) on the incorporation of ^{14}C from [^{14}C]glucose, [3-^{14}C]pyruvate and [2-^{14}C]-acetate into the soluble metabolic intermediates of chopped preparations of rat liver and brain. The work was undertaken to determine if these substances, together with salicylate, share any common actions on tissue metabolism which may possibly be related to their effects against inflammatory processes. A preliminary account of this work has been published (Moses & Smith, 1960).

EXPERIMENTAL

The techniques used were those described by Smith & Moses (1960) with the following modifications, which were designed to reduce the amounts of radioactive substrate and X-ray film used in each experiment and also to increase the ratio of labelled substrate to tissue in the incubation mixtures. Portions (0·25 ml.) of the final chopped-tissue suspension (containing either 7·2 mg. dry wt. of liver or 0·95 mg. dry wt. of brain) were dispensed into tubes (8·0 cm. length; 1·4 cm. diam.). Each tube also contained 50 μl. of the incubation medium described by Smith & Moses (1960) in the control experiments, or 50 μl. of a solution of one of the drugs in this medium. The concentrations of the drugs, after admixture with the tissue suspensions, were 10 μg./ml. for butazolidin, chloroquine diphosphate and cortisol, and 0·5 μg./ml. for dexamethasone. A solution of the radioactive substrate (5 μl.) was then added as [^{14}C]glucose (5 μc, 0·078 μmole), [3-^{14}C]-pyruvate (5 μc, 1·38 μmoles) or [2-^{14}C]acetate (5 μc, 1·01 μmoles); labelled pyruvate was not used with the brain preparations. The radioactive materials were obtained from The Radiochemical Centre, Amersham, Bucks. After incubation of the tubes for 3 hr. at 37° in an atmosphere of air, 1 ml. of absolute ethanol was added to each tube and the mixture was heated to boiling, cooled and centrifuged for 5 min. at about 1600 *g*. The supernatant was retained and the residue extracted twice by the addition of portions (0·3 ml.) of boiling 20% (v/v) ethanol, followed by centrifuging. The combined supernatants were made up to a volume of 2 ml. with absolute ethanol and portions (0·4 ml.) were analysed for the radioactive substances present by the techniques described previously (Smith & Moses, 1960). Separate analyses of the cell extract and incubation medium were not performed since the earlier work had shown that there was little, if any, difference between their patterns of radioactive intermediates. Phosphate esters were not analysed separately as they did not contain sufficient radioactivity to justify a detailed analysis.

RESULTS

Total incorporation of ^{14}C from glucose, pyruvate and acetate into soluble intermediates of liver and brain. The results given in Table 1 show that although there was no consistent effect of the drugs on the total incorporation of ^{14}C into the soluble metabolites by liver from [^{14}C]glucose, all the substances stimulated the total uptake of the isotope from the labelled glucose by the brain, dexamethasone being the most active. There were no appreciable effects of the drugs on the total incorporation of radiocarbon from [3-^{14}C]pyruvate by liver, but the corresponding uptakes from the labelled acetate were inhibited in both tissues, dexamethasone again being the most active substance.

Distribution of ^{14}C in the soluble intermediates. The results in Tables 2 and 3 show the amounts of ^{14}C incorporated into the soluble intermediates of the liver from labelled pyruvate and acetate, and into those of the brain from the labelled glucose and acetate, in the presence and absence of the four drugs.

Table 1. *Effects of butazolidin, chloroquine diphosphate, cortisol and dexamethasone on the total incorporation of ^{14}C from [^{14}C]glucose, [3-^{14}C]pyruvate and [2-^{14}C]acetate into the soluble intermediates of rat liver and brain*

Each tube contained 0·25 ml. of tissue suspension (7·2 mg. dry wt. of liver or 0·95 mg. dry wt. of brain) in the presence or absence of 10 μg./ml. of butazolidin, chloroquine diphosphate or cortisol, or of 0·5 μg./ml. of dexamethasone. The tissue was incubated with 5 μc of labelled substrate for 3 hr. at 37°. One-fifth of the total volume of extract was used for chromatography on each sheet of paper and the total counts/min. in each extract were obtained from the sum of the counts of all the radioactive spots on the paper excluding that due to residual substrate. Results are expressed as percentages of the ^{14}C incorporated into the sum of all the soluble intermediates compared with the values observed in the absence of the drugs.

	Liver			Brain	
Drug	[^{14}C]Glucose	[3-^{14}C]Pyruvate	[2-^{14}C]Acetate	[^{14}C]Glucose	[2-^{14}C]Acetate
Butazolidin	108	95	89	119	106
Chloroquine diphosphate	117	114	72	120	84
Cortisol	98	100	87	121	82
Dexamethasone	94	94	60	137	77
10^{-3} × Counts/min./25 mg. dry wt. of tissue in absence of drugs	150	830	290	550	400

Table 2. *Metabolism of* [3-^{14}C]*pyruvate and* [2-^{14}C]*acetate by rat liver in the presence and absence of four anti-inflammatory drugs*

Each tube contained 7·2 mg. dry wt. of liver in a total volume of 0·305 ml. The anti-inflammatory drugs were present in the following concentrations: butazolidin, chloroquine diphosphate and cortisol, 10 μg./ml.; dexamethasone, 0·5 μg./ml. The tissue was incubated with 5 μc of labelled substrate for 3 hr. at 37°. The Table shows the ^{14}C present in the whole sample, expressed as 10^{-3} × counts/min./25 mg. dry wt. of tissue.

Labelled substrate	[3-^{14}C]Pyruvate					[2-^{14}C]Acetate				
Anti-inflammatory drug ...	None	Buta-zolidin	Chloroquine	Cortisol	Dexa-methasone	None	Buta-zolidin	Chloroquine	Cortisol	Dexa-methasone
Sugar phosphates and uridine diphosphate glucose	3·1	2·6	2·0	2·4	3·2	4·8	4·6	2·6	2·8	2·0
Phosphoglyceric acid	0·9	0·9	0·9	0·9	0·5	0·5	0·6	0·1	0·4	0·4
Phosphoenolpyruvic acid	1·2	1·6	1·8	1·1	2·1	0·3	0·4	0·1	0·2	0·2
Nucleotides	1·6	1·1	0·2	0·5	0·6	1·9	1·8	1·7	1·4	1·1
Maltose	0	0	0	0	0	1·5	1·0	0·8	1·1	0
Aspartic acid	20	12	16	21	15	33	26	20	29	17
Glutamic acid	230	160	210	220	170	84	83	67	76	56
Alanine	310	270	340	270	230	8·4	9·3	6·5	10	6·8
γ-Aminobutyric acid	11	8·4	5·8	11	17	18	16	11	14	7·9
Glutamine	33	32	17	31	32	30	31	22	30	22
Lactic acid	130	190	190	180	230	70	54	51	56	42
Succinic acid	8·7	19	21	8·1	12	3·4	2·3	2·0	2·8	2·2
Malic acid	0	0	0·2	0	0	13	11	8·3	13	8·4
Citric acid	1·0	2·0	1·2	1·0	1·4	0·5	0·5	0·3	0·6	0·2
Glyceric acid	28	17	32	16	16	0	0	0	0	0
Total in:										
Sugar phosphates	5·2	5·1	4·7	4·4	5·8	5·6	5·6	2·8	3·4	2·5
Nucleotides	1·6	1·1	0·2	0·5	0·6	1·9	1·8	1·7	1·4	1·1
Maltose	0	0	0	0	0	1·5	1·0	0·8	1·1	0
Amino acids	610	500	620	580	480	190	180	140	170	120
Organic acids	190	260	280	220	260	87	68	62	72	53
Other substances	30	32	41	33	32	4·0	2·0	0·6	2·3	1·5
Soluble extract	830	800	950	830	780	290	260	210	250	170

Table 3. *Metabolism of* [^{14}C]*glucose and* [2-^{14}C]*acetate by rat brain in the presence and absence of four anti-inflammatory drugs*

Table 3. *Metabolism of [^{14}C]glucose and [2-^{14}C]acetate by rat brain in the presence and absence of four anti-inflammatory drugs*

Details were as given for Table 2, except that each tube contained 0·95 mg. dry wt. of brain.

Labelled substrate	[^{14}C]Glucose					[2-^{14}C]Acetate				
Anti-inflammatory drug	None	Butazolidin	Chloroquine	Cortisol	Dexamethasone	None	Butazolidin	Chloroquine	Cortisol	Dexamethasone
Sugar phosphates, uridine diphosphate glucose and nucleotides	150	180	250	240	240	0	0	0	0	0
Aspartic acid	1200	1300	1300	1300	1400	140	160	110	100	98
Glutamic acid	980	1100	1100	1200	1300	110	120	110	120	110
Alanine	900	880	950	1100	1300	9·5	9·5	4·1	0	0
γ-Aminobutyric acid	440	590	600	500	730	40	48	33	32	35
Glutamine	580	700	680	710	780	0	0	0	0	0
Lactic acid	34	96	83	46	21	3·9	0	0	0	0
Succinic acid	330	400	300	240	82	4·6	3·8	6·6	6·3	6·3
Malic acid	140	200	200	200	240	36	30	12	21	21
Citric acid	490	520	410	590	610	34	35	28	22	25
Total in										
Phosphate esters	150	180	250	240	240	0	0	0	0	0
Amino acids	4300	4700	4800	5100	5900	300	340	260	260	240
Organic acids	990	1200	990	1100	960	78	69	46	54	53
Other substances	110	450	510	350	470	22	22	35	19	14
Soluble extract	5500	6500	6500	6700	7500	400	430	340	330	310

None of the drugs affected the percentage distribution of radioactivity in the principal components of the soluble intermediates derived from these labelled substrates in the two tissues. However, all four anti-inflammatory substances showed marked effects on the distribution of radiocarbon among the soluble intermediates of liver incubated with [^{14}C]glucose (Table 4). The incorporation of the isotope into alanine, which was the most prominently labelled substance in the corresponding control experiment, was greatly decreased in the presence of the drugs, whereas increased amounts of radioactivity were found in maltose, glyceric acid, phosphoglyceric acid and lactic acid. In addition, ^{14}C was detected in glutamic acid and nucleotides in the presence of the drugs, whereas it was not observed in these compounds in the control sample. In this tissue approximately 94% of the labelled glucose was recovered unchanged at the end of the experiment in the presence or absence of the drugs.

DISCUSSION

The results show that butazolidin, chloroquine diphosphate, cortisol and dexamethasone produced very similar effects on the incorporation of ^{14}C from labelled substrates into the soluble intermediates of rat liver and brain. These four drugs, of diverse chemical structure, therefore share both anti-inflammatory properties and common actions on intermediary metabolism. However, when the present results are compared with those obtained with salicylate in similar experiments (Smith & Moses, 1960), it is apparent that there are few points of similarity. All the drugs used in the present work, as well as salicylate, caused an increased total incorporation of ^{14}C from labelled glucose into the soluble intermediates of brain, and decreased the uptake of radioactivity from [2-^{14}C]-acetate in both liver and brain. The inhibitory effects of salicylate in the latter instance were much more pronounced than with the other four drugs. The most striking differences were concerned with the distribution of ^{14}C among the various metabolic intermediates. Thus salicylate produced little, if any, effect on the distribution of the isotope in liver incubated with [^{14}C]glucose, whereas the other drugs caused a considerable decrease in the amount of radiocarbon in alanine, and this was accompanied by a relative accumulation of ^{14}C in maltose, phosphoglycerate and glycerate. In liver incubated with [3-^{14}C]pyruvate, salicylate increased the incorporation of radioactivity into glutamic acid and decreased its entry into alanine (A. K. Huggins, M. J. H. Smith & V. Moses, unpublished work); none of the other drugs had any effect in this system. It must therefore be concluded that the action of salicylate on tissue

Table 4. *Metabolism of [^{14}C]glucose by rat liver in the presence and absence of four anti-inflammatory drugs*

Details were as given for Table 2.

Anti-inflammatory drug ...	None	Butazolidin	Chloroquine	Cortisol	Dexamethasone
Sugar phosphates and uridine diphosphate glucose	13	15	14	11	13
Phosphoglyceric acid	0·8	4·0	3·9	3·6	2·3
Phosphoenolpyruvic acid	0	0	0	0	0
Nucleotides	0	11	13	12	11
Maltose	9·8	57	66	53	52
Aspartic acid	8·6	13	14	8·9	12
Glutamic acid	0	1·3	2·2	2·2	2·2
Alanine	98	18	19	16	11
γ-Aminobutyric acid	7·3	7·2	9·7	7·0	7·0
Glutamine	0	0	0	0	0·9
Lactic acid	0·8	3·7	6·2	4·9	9·1
Succinic acid	0·2	0·9	0·7	0·7	0
Malic acid	0·2	0·4	0·9	0·9	0·7
Citric acid	0	0	0	0	0·6
Glyceric acid	4·5	20	19	19	11
Total in					
Sugar phosphates	14	19	18	15	15
Nucleotides	0	11	13	12	11
Maltose	9·8	57	66	53	52
Amino acids	120	45	49	40	40
Organic acids	5·7	25	27	25	21
Other substances	1·9	4·3	1·7	1·0	1·1
Soluble extract	150	160	170	150	140

metabolism differs from that of the other four anti-inflammatory compounds.

The most prominent effects of butazolidine, chloroquine diphosphate, cortisol and dexamethasone observed in the present work were concerned with the distribution of ^{14}C in the soluble intermediates of rat liver incubated with [^{14}C]glucose. The principal changes were a greatly reduced incorporation of labelled carbon into alanine and an increased accumulation of radioactivity in lactate, phosphoglycerate, glycerate, maltose, glutamate and nucleotides. [^{14}C]Alanine was the major component of the soluble intermediates of the liver when incubated in the absence of the drugs, which suggests that transamination is an important mechanism for the further metabolism of pyruvate derived from the labelled glucose used as substrate. Thus one possible explanation of the results reported above is that the four anti-inflammatory drugs interfered with this transamination reaction, thereby decreasing the incorporation of radioactivity into alanine and increasing the metabolism of pyruvate by other pathways. Alternative mechanisms are that either the utilization of the radioactive alanine is stimulated by the drugs or the ^{14}C in the substrate or in one or more of the metabolic intermediates is diluted by endogenous material, e.g. that derived from glycogen or protein breakdown, produced in the presence of the drugs. Such effects could have resulted in increased amounts of isotope in lactate by the lactic-dehydrogenase reaction, in phosphoglycerate and glycerate due to an accumulation of glycolytic intermediates, and in glutamate by an increased entry of the pyruvate carbon atoms into the tricarboxylic acid cycle. The increased accumulation of radiocarbon in the maltose fraction may be associated with the stimulation of liver-glycogen synthesis by cortisol and other steroids described by Winters & Morrill (1955) in whole animals, and by Chiu (1950) in slices: no information is available about similar effects of butazolidin or chloroquine on liver-glycogen formation. The significance of the increased incorporation of ^{14}C into the nucleotide fractions in the presence of the drugs cannot be assessed, since the intramolecular distribution of the isotope in these substances was not studied.

In contrast with the results obtained with liver and [^{14}C]glucose, the incorporation of radioactivity into alanine was not affected by any of the drugs if either [3-^{14}C]pyruvate or [2-^{14}C]acetate was used as substrate. Thus whatever may be the mechanism concerned with the decreased radioactivity found in alanine in the presence of the drugs, it possesses a drug sensitivity which varies with the labelled substrate. These findings suggest the possibility that there may be more than one metabolic pool of substances, such as pyruvate, which are concerned with alanine metabolism in liver cells.

SUMMARY

1. The effects of butazolidin, chloroquine diphosphate, cortisol and dexamethasone on the incorporation of ^{14}C from labelled substrates into the soluble intermediates of chopped preparations of rat liver and brain have been studied.

2. All the drugs increased the total incorporation of radioactivity from [^{14}C]glucose in brain and decreased the total incorporation of the isotope from [2-^{14}C]acetate in liver and brain. The total incorporation of ^{14}C from [^{14}C]glucose and [3-^{14}C]-pyruvate in liver was not materially altered by the presence of the drugs.

3. The distribution of ^{14}C from [^{14}C]glucose in the soluble intermediates of liver was affected by all the drugs. The main changes were a greatly reduced incorporation of the isotope into alanine and an increased accumulation of radioactivity in maltose, phosphoglycerate, glycerate, glutamate and lactate. None of the drugs affected the distribution of ^{14}C from [^{14}C]glucose in brain, from [3-^{14}C]-pyruvate in liver or from [2-^{14}C]acetate in both liver and brain.

4. Some implications of these results are discussed.

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Biosynthesis of Polynucleotides

4. THE UTILIZATION OF EXOGENOUS PRECURSORS BY *CANDIDA UTILIS**

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The presence of the enzyme polynucleotide phosphorylase in bacteria and in plant and mammalian tissues (Grunberg-Manago, Ortiz & Ochoa, 1956; Beers, 1957; Brummond, Staehelin & Ochoa, 1957; Littauer & Kornberg, 1957; Hilmoe & Heppel, 1957; Hakim, 1959) has made it appear likely that nucleoside 5′-diphosphates are the immediate precursors in the biosynthesis of ribonucleic acid. Cell-free preparations have also been obtained which catalyse the incorporation of nucleoside 5′-triphosphates into ribonucleic acid (Cannellakis, 1957; Edmonds & Abrams, 1957; Hecht, Zamecnik, Stephenson & Scott, 1958; Chung & Mahler, 1958). Since nucleoside diphosphates and triphosphates are readily interconvertible in most tissues, it is difficult to distinguish between them as precursors of ribonucleic acid *in vivo*, or in tissue homogenates. However, it seems probable that incorporation of nucleoside 5′-monophosphates into ribonucleic acid (Goldwasser, 1955; Heidelberger, Harbers, Leibman, Takagi & Potter, 1956) takes place through either the corresponding 5′-diphosphates or 5′-triphosphates. In part 1 of this series (Barker, Montague, Moss & Parsons, 1957) it was shown that, *in vitro*, pancreatic ribonuclease, besides catalysing the formation of ester linkages between simple nucleotides (Heppel, Whitfeld & Markham, 1955), can also bring about the attachment of nucleotides to ribonucleic acid. Such a synthetic reaction taking place *in vivo* might manifest itself in the incorporation of nucleoside 3′- (or 2′-)phosphates into ribonucleic acid through the corresponding cyclic phosphates. It is clearly desirable to establish whether this biosynthetic route does in fact occur in tissues in any circumstances, and experiments are in progress to test this possibility. The work reported here is an attempt to determine the extent to which new internucleotide bonds of ribonucleic acid are formed by polymerization of nucleoside 5′-phosphates. In the present experi-

* Part 3: Barker & Foll (1957).

ments incorporation of exogenous precursors into the ribonucleic acid of *Candida utilis* growing exponentially has been studied.

METHODS

Measurement of radioactivity. Samples of aqueous solutions of materials under investigation were evaporated on aluminium planchets and counted at infinite thinness with a thin end-window counter (window thickness, 1·7 mg./cm.^{2}). Counting was continued until the statistical error was between 5 and 10%. Samples containing both ^{14}C and ^{32}P were also counted with an aluminium shield of thickness 12·6 mg./cm.^{2} which absorbed 97% of ^{14}C-emission and 6% of ^{32}P-emission. Corrections were applied for decay of ^{32}P and for absorption by the aluminium shield. Concentrations of solutions for assay were determined by measurement of extinction at 260 mμ where appropriate; concentrations of solutions of ribose were determined by Euler & Hahn's (1946) method.

Electrophoresis. Paper electrophoresis was carried out by the method of Markham & Smith (1952) with citrate or acetate buffer at pH 3·5 and a voltage gradient of 20 v/cm. Authentic materials were examined simultaneously with experimental samples.

Paper chromatography. The following solvent systems were used: solvent 1, 5% Na_2HPO_4–*iso*pentanol (ascending) (Carter, 1950); solvent 2, propan-2-ol–water–aq. 28% NH_3 soln. (85:15:1·3, by vol.) (descending) (Hershey, Dixon & Chase, 1953); solvent 3, butan-1-ol–water–formic acid (77:13:10, by vol.) (descending) (Markham & Smith, 1949); solvent 4, propan-2-ol–water–saturated $(NH_4)_2SO_4$ (2:19:79) (Deutsch & Nilsson, 1953). Phosphate was detected by the method of Wade & Morgan (1953) and ribose by the method of Buchanan, Dekker & Long (1950). Authentic materials were chromatographed on the same paper strips as the experimental samples.

Ion-exchange-resin chromatography. Mixtures of nucleotides were chromatographed by a modification of the method of Cohn (1950) with columns (15 cm. × 0·5 cm. or 15 cm. × 1 cm.) of Dowex-2 resin (formate form, 8% cross-linked, 200–400 mesh). The material to be chromatographed (approx. 5 mg. of total nucleotides for the small columns) was dissolved in 20 ml. of water, and the pH was adjusted to 8·5 with aq. NH_3 soln. Nucleotides were washed from the column by gradient elution (flow rate 1 ml./min. and 0·5 ml./min. for the larger and smaller columns respectively) by using 4N-formic acid and a 500 ml. mixing flask containing water initially. Fractions (5 ml.) were collected and their extinctions at 260 mμ were measured.

Growth of organism. C. utilis (National Collection of Industrial Bacteria; IMI. 23311) was grown in a medium containing (g./l.): glucose, 20; $(NH_4)_2SO_4$, 4·5; KH_2PO_4, 2·0; $MgSO_4,7H_2O$, 0·5; $CaCl_2$, 0·25; the pH was 4·8. In experiments involving incorporation of [^{32}P]orthophosphate, the concentration of KH_2PO_4 was decreased to 10 mg./l. Cultures were maintained on agar slopes and were subcultured every 3 weeks. Incorporation experiments were carried out with exponentially growing cells as follows. The liquid medium (150 ml.) was inoculated from a slope and incubated at 30° overnight with vigorous aeration. The cells were harvested by centrifuging and suspended in fresh medium to which the material to be incorporated had been added, such that the cell suspension gave a reading of 20 in the EEL (Evans Electroselenium Ltd., Harlow, Essex) colorimeter used with a red filter. In preparative incorporation experiments, incubation with the fresh medium was continued until growth ceased; in other experiments incorporation was allowed to proceed either until the colorimeter reading was 40 (approx. 90 min.) or, in some experiments as indicated, for 15 min. In all experiments, the cells were harvested by centrifuging and were washed once with a volume of 0·14M-NaCl equal to that of the medium. In experiments in which the radioactivity of the acid-soluble fraction was measured, the cells were washed with three further quantities of 0·14M-NaCl, when a sample (1 ml.) of the supernatant was found to have no measurable activity.

Extraction of sodium nucleates. Experiments in which Kirby's (1956) method was used resulted in extraction of only traces of ribonucleic acid (RNA) from the yeast. A modification of the method of Schmidt & Thannhauser (1945) was therefore used. Since it was intended to hydrolyse the material to nucleoside 5′-phosphates, treatment with alkali was omitted and it was not possible to remove all deoxyribonucleic acid (DNA). However, experiments showed that the sodium nucleates extracted as described below were contaminated with not more than small traces of DNA, which were ignored (cf. Mitchison & Walker, 1958).

The washed yeast was extracted at 0° overnight with 5 vol. of 7·5% (w/v) trichloroacetic acid. The acid-soluble material was obtained as a supernatant after centrifuging, and the residue was washed twice with 7·5% (w/v) trichloroacetic acid. For radioactive measurements trichloroacetic acid was removed by extraction with ether. The residue from which the acid-soluble fraction had been removed was boiled for 2 hr. under reflux with 5 vol. of ethanol–ether (3:1, v/v) and once similarly with methanol–$CHCl_3$ (1:1, v/v). The residue from this treatment was heated twice at 90° for 1 hr. with 10 vol. of 10% (w/v) NaCl. To the combined supernatants obtained by centrifuging, ethanol (3 vol.) was added, and the precipitate was collected by centrifuging again, washed with ethanol and dried over $CaCl_2$ (RNA fraction). The yield was approx. 30 mg./g. of dry yeast.

Preparation of nucleoside 5′-phosphates from the ribonucleic acid fraction. A solution of sodium nucleate (100 mg.) in water (100 ml.) was brought to pH 9·3 with aq. 2N-NH_3 soln. and incubated at 37° for 18 hr. with phosphodiesterase prepared from Russell's viper's venom (Ross Allen's Reptile Institute, Silver Springs, Florida, U.S.A.) (20 mg. of venom) by the method of Hurst & Butler (1951). Perchloric acid (30%, w/v) was added to give pH 1, and, after centrifuging, the supernatant was adjusted to pH 8·5 with 0·3N-KOH and chromatographed on a column (1 cm. diameter) of Dowex-2 resin. Appropriate fractions were combined, evaporated below 35°, and finally freeze-dried. Four components were obtained which were characterized respectively as cytidine 5′-phosphate, adenosine 5′-phosphate (AMP), guanosine 5′-phosphate and uridine 5′-phosphate spectroscopically, by paper electrophoresis and by paper chromatography before and after treatment with the 5′-nucleotidase of crude Russell's viper's venom.

Preparation of nucleoside 2′- and 3′-phosphate from the ribonucleic acid fraction. These materials were prepared by

alkaline hydrolysis and ion-exchange chromatography by the method of Cohn (1950).

Preparation of [8-^{14}C]*adenosine.* [8-^{14}C]Adenine (The Radiochemical Centre, Amersham, Bucks.) was converted into N^6-acetyladenine-9-mercurichloride, which was condensed with 2:3:5-tri-*O*-benzoyl-D-ribofuranosyl chloride by a method analogous to that of Fox, Yung, Davoll & Brown (1956). After removal of protecting groups, the material was purified by crystallization from water. The material gave single spots on paper chromatograms run in solvents 1 and 2.

Preparation of [8-^{14}C]*adenosine phosphates*

Isotopically-labelled nucleotides were obtained either synthetically from [8-^{14}C]adenosine, or biosynthetically from [8-^{14}C]adenine.

Synthesis. Owing to the small scale on which experiments were carried out, it was found more economical to phosphorylate adenosine itself rather than its *iso*propylidene derivative. This has the disadvantage that some 2′- and 3′-phosphates are also formed, but these were decreased to a minimum by phosphorylating with dibenzyl phosphorochloridate under scrupulously anhydrous conditions (cf. Barker & Foll, 1957), about two-thirds of the total yield of nucleotides consisting of the 5′-phosphate.

Dibenzyl phosphorochloridate prepared from dibenzyl phosphite (0·18 ml.) was added to a solution of [8-^{14}C]-adenosine (20 mg.) in 4 ml. of pyridine (dried immediately before use by distillation from P_2O_5) at $-40°$. The mixture was maintained at $-40°$ in a stoppered flask for 3 hr., and the temperature was then allowed to rise to room temperature overnight. Saturated aq. Na_2CO_3 (1·5 ml.) was added with shaking, and solvents were then removed under reduced pressure below 35°. The residue was extracted with $CHCl_3$, the extract was washed with aq. 1% (w/v) $NaHCO_3$ (0·5 ml.) and with water (0·5 ml.), dried over Na_2SO_4 and evaporated to dryness under reduced pressure with five successive portions of ethanol (10 ml.). The residue was dissolved in aq. 50% (w/v) ethanol (12 ml.) and hydrogenated at just above atmospheric pressure in the presence of 10% of palladium–charcoal catalyst. After removal of solvent and catalyst, the residue was dissolved in water (10 ml.), the pH was adjusted to 8·5 with aq. NH_3 soln. and the solution was chromatographed on a column (diameter 1 cm.) of Dowex-2 resin. Three fractions were obtained which, after evaporation under reduced pressure followed by freeze-drying, yielded respectively AMP, adenosine 2′- and adenosine 3′-phosphate, identified by comparison with authentic samples on chromatograms run in solvent 4. A sample of the AMP was also converted into adenosine by Russell's viper's venom as described above. Before use, the labelled nucleotides were rechromatographed, and the purity was checked by comparison of the area of the paper containing radioactive and ultraviolet-absorbing materials.

Biosynthesis. C. utilis was grown in a medium containing a supplement of 0·1 mc of adenine in each 150 ml. of stock medium. The sodium nucleates were extracted and hydrolysed as described above. The AMP obtained was shown to contain not less than 97·2% of its radioactivity in the adenine residue as follows. A sample (approx. 1 mg.; 2880 counts/min.) of the material was dissolved in water (5 ml.) and 5 ml. of damp Dowex-50 resin (H^+ form) was added. After 5 min., the resin was collected by filtration, washed with water and heated at 100° for 3 min. with water (5 ml.). After cooling, the resin was collected by filtration and washed with water. The filtrate was chromatographed in solvent 1 and shown to contain D-ribose 5-phosphate but no adenine or adenine-containing material. The solution containing the ribose phosphate was transferred to a planchet and gave 80 counts/min. The resin was eluted with aq. 2N-NH_3 soln., and the filtrate was chromatographed in solvent 1. It gave no test for ribose-containing material and showed only one spot of ultraviolet-absorbing material, corresponding to adenine. The solution was evaporated and transferred to a planchet and gave 2800 counts/min. The residual resin was not radioactive.

Generally labelled [^{14}C]*adenosine 5′-phosphate. C. utilis* was grown in 150 ml. of normal medium containing 0·1 mc of generally labelled [^{14}C]glucose (The Radiochemical Centre), and AMP was prepared as described above. The radioactivity in the adenine and ribosyl residues was determined by the method of Roll, Weinfeld, Carroll & Brown (1956); 12·1% of the activity was present in the adenine residue.

[^{32}P]*Adenosine 5′-phosphate. C. utilis* was grown in normal medium containing 10^6 counts/min./l. of $KH_2{}^{32}PO_4$. The AMP was prepared and characterized chromatographically as described above.

[^{32}P$^{\alpha}$]*Adenosine 5′-diphosphate.* [^{32}P]AMP was condensed with non-radioactive orthophosphate by the method of Lowenstein (1957). The product was chromatographed on Dowex-2 resin, fractions being obtained which corresponded with AMP and adenosine 5′-diphosphate (ADP); a small fraction containing adenosine 5′-triphosphate (ATP) was also obtained. These materials were identified by comparison with authentic samples on electrophoresis in acetate buffer. Samples of the solutions containing the diphosphate and triphosphate were made 0·1N with respect to HCl and heated at 90° for 15 min. The hydrolysed material was then again submitted to electrophoresis and gave spots corresponding with AMP. The ADP and ATP were purified before use by electrophoresis. The materials were eluted with water from appropriate areas of the paper and freeze-dried.

RESULTS

Extent of utilization of exogenous precursors. The results are presented in Table 1. It is seen that, of the compounds examined, only adenine is efficiently utilized for RNA synthesis, approx. 60% of the label being incorporated into the RNA fraction. ADP and ATP were incorporated to a small but significant extent. In all three cases, incorporation into the RNA fraction was greater than that into the acid-soluble fraction. No detectable activity was found in either fraction of cells grown for 90 min. in a medium supplemented with [8-^{14}C]-adenosine or its three isomeric monophosphates. No measurable activity was detected in whole, washed cells grown under these conditions, suggesting that, after the cells had been washed, not more than approx. 0·005% of the adenosine, or 0·035% of the monophosphates, remained in the

cells. It is believed that the radioactivity of the RNA fraction properly represents incorporation into the RNA molecule since, after incorporation of [8-^{14}C]adenine for preparative purposes, radioactive nucleoside 5′-phosphates were obtained after enzymic hydrolysis of the RNA, whereas alkaline hydrolysis yielded radioactive nucleoside 3′-phosphates. Furthermore, after incorporation of the isotope of labelled ADP, radioactive AMP was recovered from the RNA, whereas no activity was found in the AMP fraction of RNA after incubating the yeast with isotopically labelled AMP.

Equilibration of isotopic labels with metabolic pools. Experiments were carried out over a period of one generation (90 min.). Tables 2 and 3 show the results obtained with [^{32}P]ADP mixed with [8-^{14}C]ADP and [^{32}P]ADP mixed with generally labelled [^{14}C]ADP. It is seen that, under these conditions of growth, the phosphorus of all four nucleoside 5′-phosphates derived from the RNA becomes labelled. ^{14}C appears in both adenine and guanine residues. No measurable activity was found in the ribosyl residues of any of the nucleotides with either generally or specifically labelled precursors.

Initial utilization of adenosine 5′-di- and triphosphate for ribonucleic acid synthesis. In order to investigate the extent to which added ADP and ATP are used as such for RNA synthesis, incorpora-

Table 1. *Incorporation of* [8-^{14}C]*adenine and derivatives into the acid-soluble and ribonucleic acid fractions of exponentially growing* Candida utilis

C. utilis was grown for 90 min. in normal medium (150 ml.) to which the appropriate radioactive supplement had been added (approx. 50 μg.). Acid-soluble and RNA fractions were isolated as described in the Methods section, the whole of each fraction was transferred to planchets and the radioactivity measured.

Supplement	Radioactivity (counts/min.) Total added	Acid-soluble fraction	RNA
Adenine	1000	62	645
Adenosine	6420	$\gtrless$0·35	$\gtrless$0·35
Mixed adenosine 2′- and 3′-phosphates	1000	$\gtrless$0·35	$\gtrless$0·35
AMP	1000	$\gtrless$0·35	$\gtrless$0·35
ADP	832	1·3	4·5
ATP	591	2·7	5·5

Table 2. *Incorporation of doubly labelled adenosine 5′-diphosphate into ribonucleic acid of exponentially growing* Candida utilis

C. utilis was grown for 90 min. on normal medium (150 ml.) to which a supplement consisting of a mixture (approx. 50 μg.) of [8-^{14}C]ADP and [^{32}P]ADP had been added. RNA was isolated and hydrolysed as described in the Methods section to nucleoside 5′-phosphates, which were examined for radioactivity due to ^{14}C and ^{32}P. GMP, Guanosine monophosphate; UMP, uridine monophosphate; CMP, cytosine monophosphate.

Nucleotide examined	Radioactivity (counts/min./μmole) ^{14}C	^{32}P	Ratio of activities ^{14}C/^{32}P
ADP (supplement)	493$\pm$0·1	164$\pm$0·1	3·0$\pm$0·002
AMP	36·9$\pm$0·37	1·9 $\pm$0·19	19·4$\pm$1·9
GMP	28·4$\pm$0·37	1·36$\pm$0·16	20·8$\pm$2·5
UMP	$\gtrless$0·35	3·5 $\pm$0·98	$\gtrless$0·35
CMP	$\gtrless$0·35	0·7 $\pm$0·3	$\gtrless$0·35

Table 3. *Incorporation into ribonucleic acid of exponentially growing* Candida utilis *of* [^{32}P]- *and generally labelled* [^{14}C]-*adenosine 5′-diphosphate*

C. utilis was grown for 90 min. on normal medium (150 ml.) to which a mixture (approx. 50 μg.) of [^{32}P]ADP and generally labelled [^{14}C]ADP had been added. RNA was isolated and hydrolysed as described in the Methods section to nucleoside 5′-phosphates, which were examined for radioactivity due to ^{14}C and ^{32}P. The purine nucleotides were also hydrolysed and the radioactivity of the purine base was determined as described in the Methods section. Abbreviations are as in Table 2.

Nucleotide examined	Radioactivity (counts/min./μmole) ^{14}C Total	^{14}C Purine	^{14}C Ribose	^{32}P	Ratio of activities ^{14}C(purine)/^{32}P
ADP (supplement)	700·8 $\pm$0·1	166·6 $\pm$0·1	543·2$\pm$0·1	450$\pm$0·1	0·37$\pm$0·0002
AMP	0·92 $\pm$0·037	0·79$\pm$0·048	$\gtrless$0·35	0·3 $\pm$0·019	2·64$\pm$0·2
GMP	0·49 $\pm$0·059	0·47$\pm$0·1	$\gtrless$0·35	0·166$\pm$0·05	2·94$\pm$0·89
CMP	0·11 $\pm$0·098	—	—	0·2 $\pm$0·07	—
UMP	0·084$\pm$0·05	—	—	0·23 $\pm$0·04	—

Table 4. *Suppression of incorporation of* [^{32}P]*orthophosphate into nucleotides derived from ribonucleic acid of* Candida utilis *by adenosine 5′-di- and -tri-phosphate*

C. utilis was grown for 15 min. in normal medium (150 ml.) to which supplements of either [^{32}P]orthophosphate (approx. 150 μc, carrier-free) alone or together with the nucleotide under investigation (0·1 m-mole) had been added. RNA was isolated and hydrolysed either by snake-venom diesterase or by alkali, the nucleotides were separated as described in the Methods section, and the radioactivity was measured. The specific activities of the nucleotides relative to the guanine nucleotides are recorded. The results are the mean of three experiments in each case.

Supplement other than [^{32}P]ortho-phosphate	Relative specific activity (activity of guanine nucleotide taken as unity)					
	Nucleoside 5′-phosphate derived from			Nucleoside 2′- and 3′-phosphate derived from		
	Adenine	Cytosine	Uracil	Adenine	Cytosine	Uracil
None	1·23±0·03	0·92±0·03	1·46±0·03	1·28±0·02	1·15±0·03	1·14±0·04
Adenine	1·26±0·03	0·95±0·03	1·47±0·03	—	—	—
Adenosine	1·23±0·03	0·90±0·03	1·45±0·03	—	—	—
AMP	1·23±0·03	0·93±0·03	1·46±0·01	1·27±0·03	1·21±0·03	1·17±0·03
ADP	0·52±0·03	0·91±0·03	1·45±0·03	0·85±0·03	0·96±0·03	1·00±0·03
ATP	0·33±0·03	0·90±0·01	1·43±0·02	0·79±0·01	0·90±0·02	0·96±0·01

tion was investigated over a brief period of time (15 min.). Owing to the low incorporation of ADP and ATP, it was not possible to use precursors of sufficiently high specific activity to give reliable results. Experiments were therefore conducted in the following way. Yeast was grown in the presence of [^{32}P]orthophosphate for 15 min., the RNA was extracted, hydrolysed with snake-venom diesterase, and the radioactivity of the four nucleoside 5′-phosphates was measured. This gave a reasonably reproducible pattern of labelling (see Table 4). The yeast was then grown in the presence of both [^{32}P]-orthophosphate and the inactive precursor under investigation. The nucleoside 5′-phosphates from the RNA were again examined. The results are shown in Table 4. It is seen that addition of AMP to the medium has no significant effect on the pattern of labelling of the nucleoside 5′-phosphates. On the other hand, addition of ADP or ATP results in a decreased activity of the AMP relative to the other nucleosides, whereas the activities of the other three nucleotides relative to each other remain unchanged. Similar experiments in which the RNA was hydrolysed with alkali showed that the relative activities of the uracil and cytosine nucleotides were decreased to some extent as well as that of the adenine nucleotide.

DISCUSSION

Relative efficiency of adenine and its derivatives as precursors of ribonucleic acid

Previous studies of the utilization of exogenous precursors of RNA by micro-organisms have demonstrated that adenine is incorporated into the RNA of *C. utilis* (Kerr, Seraidarian & Brown, 1951; Kerr & Chernigoy, 1953), *Tetrahymena gelii* (Flavin & Graff, 1951) and *Lactobacillus casei* (Elion & Hitchings, 1950). In the last organism and in a *Neurospora* mutant (McNutt, 1958), adenosine is also utilized for RNA synthesis, and in *Escherichia coli* (Balis, Lark & Luzzati, 1955) adenosine 3′-phosphate is also efficiently utilized. On the other hand, in *C. utilis*, adenosine and derived nucleotides are utilized only to a very small extent (Kerr *et al.* 1951). It is seen that the results now presented are in general agreement with previous work so far as adenine is concerned. As was observed by Cowie & Bolton (1957), we also find that incorporation into RNA is greater than into the acid-soluble fraction. It should be noted that in the experiments of Kerr *et al.* (1951), in which a limited utilization of adenosine and its monophosphates was observed, incorporation was allowed to proceed for a very much longer period of time than in our experiments. Under our conditions it is possible to study the utilization of ADP since any independent utilization of adenosine or its monophosphates for RNA formation is not likely to amount to more than 7% of the utilization of ADP.

Metabolism of exogenous adenosine 5′-diphosphate

The experiments recorded in the experimental section, in which incorporation of labelled ADP was examined after 90 min. when the number of cells had doubled, give some indication of the utilization of this precursor in the complete cycle of reactions involved in RNA synthesis. [^{32}P]ADP labelled only in the α-phosphate residue was used and the results therefore relate only to the possible utilization of the AMP unit of the ADP molecule.

Since the phosphate residues of all four nucleoside 5′-phosphates obtained from the RNA were labelled, and the ratio of the specific activity of adenine to that of the phosphorus of the AMP residue of the RNA differed from that in the

exogenous ADP, it follows that the AMP residue of ADP does not appear as an intact unit in the RNA after duplication of the cellular material is complete. Moreover, the isotopic label of the ribosyl residue of the ADP supplied was so diluted that no activity could be detected. This indicates that the α-phosphate residue is brought into equilibrium with the orthophosphate (and/or pyrophosphate) pool and that there is no metabolically stable reserve of phosphorylated ribose compounds such as 5-phosphoribosyl pyrophosphate. It also follows that fission of the glycosidic linkage of the ADP is complete.

Previous workers have observed the conversion of exogenous adenine into RNA guanine in *C. utilis* (Kerr *et al.* 1951). The present results show that a similar conversion occurs in this organism when ADP is supplied. Although the ratio of the specific activity of the RNA adenine to that of RNA guanine varies from one experiment to another, which is in accord with the results of Cowie & Bolton (1957), there is no significant difference between the ratio specific activity of base/specific activity of phosphate for AMP and that for guanosine monophosphate derived from the same RNA. This is in agreement with the existence of an equilibrium between the AMP and guanosine monophosphate units of RNA and a common intermediate as suggested by Tomlinson & Zbarsky (1957). Such an intermediate might be inosinic acid since this compound is known to be convertible into both AMP and guanosine monophosphate and is common to known routes for the synthesis of these compounds. Thus the present results are in agreement with the conversion of adenine into guanine at the nucleotide level, but the same result could also arise if free adenine were converted into free guanine, provided that both the corresponding nucleotides are in equilibrium with the same phosphate pool. This point is discussed further below.

Immediate precursors of ribonucleic acid

The experiments discussed above give no evidence as to the nature of the immediate precursors of RNA. However, in experiments with a shorter period of incorporation, fewer reactions appear to be involved. Suppression of the incorporation of orthophosphate into only the AMP fraction of RNA by exogenous ADP or ATP strongly suggests that the AMP units of these compounds are rapidly incorporated as such into the RNA molecule. An alternative explanation needs consideration, namely that the exogenous nucleotides are broken down to yield adenine and orthophosphate, which would dilute the activity of the orthophosphate of the medium to approximately one-tenth of its original value. In this case, it would be necessary to postulate that the presence of adenine or adenosine so formed had a specific effect on the incorporation of orthophosphate from the medium into the adenine nucleotide fraction of the RNA. No such effect has been observed, as shown in Table 4, and it is concluded that in *C. utilis* under conditions of rapid growth, polymerization of nucleoside 5′-phosphates is a primary reaction in the formation of RNA from ADP or ATP.

It is seen from Table 4 that the activities of the 2′- and 3′-phosphate of cytidine and uridine are affected by added ADP or ATP as much as those of adenosine 2′- and 3′-phosphate. This is consistent with the view that the AMP unit is incorporated into the RNA molecule, since, after alkaline hydrolysis a phosphorus atom originally part of such a unit would remain attached, not to the original nucleoside, but to the nucleoside residue to which the AMP became joined in the process of incorporation (cf. Goldwasser, 1955).

Finally, incorporation of the phosphate residue of the precursors studied results in either all the nucleoside 5′-phosphates or only the AMP being affected, according to the period of incorporation. The absence of decreased activity in the guanosine monophosphate fraction after a brief period of incorporation may be due to polymerization of ADP or ATP being more rapid than the reactions involved in the conversion of adenine nucleotides into guanine nucleotides. On the other hand, the fact that conversion of adenine into guanine appears not to take place in the absence of equilibration of the phosphate residue with the pool strongly suggests that the process involves direct interconversion of the free bases rather than conversion at the nucleotide level.

SUMMARY

1. The incorporation of [8-^{14}C]adenine, [8-^{14}C]-adenosine, [8-^{14}C]adenosine 2′- and 3′-phosphate, [8-^{14}C]adenosine 5′-phosphate, [8-^{14}C]adenosine 5′-diphosphate and [8-^{14}C]adenosine 5′-triphosphate into the acid-soluble and ribonucleic acid fractions of rapidly growing *C. utilis* has been studied.

2. Adenosine and its monophosphates were not taken up into either fraction. The other three compounds resulted in a higher degree of labelling in the ribonucleic acid than the acid-soluble fraction.

3. Adenine was efficiently utilized in ribonucleic acid synthesis; adenosine 5′-diphosphate and 5′-triphosphate were utilized to a small but significant extent.

4. Incorporation of adenosine 5′-diphosphate, labelled in the adenine, ribosyl and phosphate residue, over a period of a generation, results in the utilization of all three residues independently.

5. In a period of 15 min., adenosine 5′-di- and -tri-phosphate, but not adenosine 5′-monophosphate, suppress the incorporation of [^{32}P] orthophosphate into only the adenosine 5′-monophosphate residues of ribonucleic acid.

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Further Observations on the Paraffins and Primary Alcohols of Plant Waxes

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In a series of papers dealing with researches carried out between 1930 and 1938 on the long-chain constituents of plant and insect waxes it was inferred, from evidence based largely on melting-point and long-crystal-spacing data, that all the saturated ketonic and hydroxy acids, as also the corresponding primary alcohols, contained an even number of carbon atoms, whereas the paraffins, ketones and secondary alcohols, which are probably formed from their respective acids by decarboxylation, contained an odd number of carbon atoms (Chibnall, Piper, Pollard, Williams & Sahai, 1934). Such findings, in so far as they related to the saturated acids and primary alcohols, seemed to bring the wax constituents into line with the views then current about the constituents of shorter chain-length present in glycerides and similar natural products. There was much less evidence at the time with respect to the *n*-paraffins. The above-mentioned workers examined the meagre

data then available and concluded that in all cases the products hitherto isolated consisted of one or more of the odd-number members from C_{25} to C_{37}. A more comprehensive investigation was carried out in 1937–38 on the paraffins present in rose-petal wax (Chibnall, Mangouri & Piper, 1954). A preliminary separation was made by vacuum-distillation and it was possible finally to obtain a series of fractions of recognizable purity showing the presence of all the odd-number members from C_{21} to C_{37}. Such of the intermediary fractions as had melting points corresponding to the even-number members of the series, however, were all shown to be complex mixtures and in no instance was definite evidence for the presence of any such member obtained.

In more recent years many new techniques have been used on the problem and the evidence so adduced casts doubt on the general validity of the odd–even relationships mentioned above. It is now well established, for instance, that small, generally very small, amounts of the odd-number fatty acids are present in certain glycerides. By column chromatography Ivanoff, Stoïanova-Ivanova & Ivanoff (1955) claim to have isolated both odd- and even-number paraffins from Bulgarian rose-petal wax, and Wanless, King & Ritter (1955), who submitted their column fractions to mass spectrography, were able to demonstrate the presence of a similar mixture in pyrethrum wax. These newer findings call for a re-examination of the materials used by Chibnall and his colleagues, whose conclusions had been based in large part on data derived from a series of synthetic paraffins of appropriate chain-length. If the glyceride fatty acids from which these had been prepared had unwittingly contained small amounts of neighbouring odd-number acids the final products could have been contaminated with nearby odd- or even-number homologues as the case may be. It has therefore been considered advisable to submit such of the earlier synthetic paraffins as are still available, as well as some of the paraffins isolated from waxes or derived from wax primary alcohols, to analysis by mass spectroscopy.

MATERIALS

Synthetic paraffins. (Main reference: Piper *et al.* 1931.) *n*-Tricosane had been prepared from a sample of Kahlbaum's purest lauric acid that had been recrystallized four times successively from large volumes of acetone. *n*-Heptacosane had been prepared from Kahlbaum's purest myristic acid treated in a similar way. A sample of myristic acid that had been fractionated as the ethyl ester to constant setting point had been used to prepare *n*-hexacosane (Garner, van Bibber & King, 1931) and (via *n*-tetradecanol) *n*-octacosane. *n*-Nonacosane had been prepared from a sample of *n*-pentadecanoic acid (Eastman Kodak Co.) that had been fractionally distilled four times successively and then repeatedly recrystallized from light petroleum (b.p. below 40°).

Paraffins isolated from plant waxes. The products from runner-bean leaves and cocksfoot have not hitherto been described. The following comments refer to Chibnall *et al.* (1934) unless otherwise stated. Candelilla wax, series no. B372; apple cuticle, series no. C136 (Chibnall, Piper, Pollard, Smith & Williams, 1931); swede turnip, series no. B379; Brussels sprout, unfractionated sample (Sahai & Chibnall, 1932); white mustard; rye grass, unfractionated sample (Pollard, Chibnall & Piper, 1931); green tobacco leaf, unfractionated sample; cactus, unfractionated sample.

Paraffins of rose-petal wax. The fractions selected for further analysis had melting points close to those of even-number paraffins. The previous work had suggested that these were all complex mixtures (Chibnall *et al.* 1954).

Paraffins derived from primary alcohols isolated from various waxes. The samples from barley and runner-bean leaves have not hitherto been described. Cocksfoot, one of several samples that collectively had been used to demonstrate that the primary alcohol was C_{26}, with small amounts of C_{24} and C_{28} (Pollard *et al.* 1931); wheat, series no. B261 (Pollard, Chibnall & Piper, 1933); apple cuticle, series no C72 (Chibnall *et al.* 1931); lac wax (bulked samples 5, 6, 7, 11, 13 and 14), wild white clover, cactus and white mustard (Chibnall *et al.* 1934); lucerne, series no. B226 (Chibnall, Williams, Latner & Piper, 1933).

METHODS

The method of chemical analysis by mass spectrometry has been described by several authors, e.g. Washburn (1950) and Waldron (1956). Only a brief account of the experimental technique will be given here.

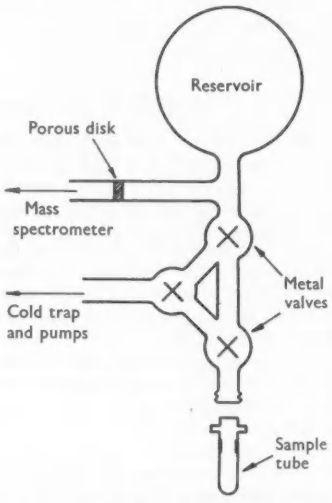

Fig. 1. Schematic diagram of heated inlet system.

The method of analysis of *n*-paraffin hydrocarbons employed in the present work closely followed that originally developed by O'Neal & Weir (1951), except that the mass spectrometer used was the Associated Electrical Industries type MS 2-H, and that a different type of sample-introduction system was employed. A detailed description of the latter has been given by Craig & Errock (1959) and a sketch is shown in Fig. 1. The main feature of this introduction system is that it enables a weighed quantity of a solid sample to be introduced from atmospheric pressure through metal valves into a heated glass reservoir. The glass sample tube (Fig. 1) can be detached from the inlet system and a melting-point tube containing the sample placed in the tube. The glass sample tube is permanently attached to a metal insert through a glass–metal seal and the whole assembly is fitted to the inlet system by means of a screw connexion and gold gasket. After pumping away the air above the tube, the sample can be evaporated directly into the reservoir. About 1 mg. of material was usually employed and the partial pressure of paraffin in the reservoir was approx. 0·05 mm. Hg. In the present work the reservoir and valves were operated at a temperature of approx. 320°, which was sufficiently high to maintain the paraffin hydrocarbon in the vapour phase but yet was sufficiently low to avoid thermal-decomposition effects. Any products of pyrolysis would have been readily detected since the mass spectrum would have been observed to change with time.

From the reservoir the sample flowed at a steady rate through a porous disk, which reduced the pressure to about 5×10^{-6} mm. Hg, and then through a glass tube heated to 150° and into the ionization chamber of the mass spectrometer. The rate of loss of sample from the reservoir was small, approx. 10%/hr. for *n*-hexadecane, which was negligible in the 5–10 min. required to scan the mass spectrum. In the ionization chamber, the molecules of hydrocarbon vapour were bombarded with electrons of 70v energy and a record of the mass spectrum of the positive ions produced was obtained in the usual way by magnetic scanning.

Two typical examples of the mass spectra obtained are reproduced in Figs. 2 and 3. The mass spectrum is essentially a plot of the relative ion intensity against mass. The height of the peak at any mass number is directly proportional to the number of positive ions of that particular mass formed in the ionization chamber, and this, in turn, is directly proportional to the partial pressure of the compound from which the ions originate. If the ions originate from more than one compound then the peak height is proportional to the sum of the partial pressures of the compounds concerned. It should be noted that the mass scale is known exactly and that, consequently, there can be no doubt about the mass assignments in any spectrum.

Interpretation of the mass spectra and method of calculation

The method is best described in terms of an example, and Fig. 2 shows the upper portion of the mass spectrum of the paraffin derived from the primary alcohol of wheat, which is almost pure *n*-octacosane.

Two types of positive ions are produced on electron bombardment, namely, parent or molecular ions and fragment ions. The molecular ions are simply those which are formed by removing one electron from the molecule; they occur at the mass corresponding to the molecular weight of the compound and the intensity of the ion current at that mass is directly proportional to the partial pressure of the compound in the reservoir. Molecular-ion peaks are designated 'm' in Figs. 2 and 3. Thus the peak at mass 394 in Fig. 2 is due to the ions $C_{28}H_{58}^+$ produced from *n*-octacosane.

Fragment ions are produced by ionization and dissociation of the neutral molecule and the most prominent in the spectrum of a *n*-paraffin are the alkyl ions, $C_nH_{2n+1}^+$, designated 'a' in Figs. 2 and 3. These are unimportant from the point of view of quantitative analysis, but, qualitatively, they serve to distinguish a *n*-paraffin from other compounds of the same molecular weight. The regular pattern of alkyl-ion peaks in Fig. 2, increasing in intensity towards lower mass, is quite characteristic of *n*-paraffins; ketones or *iso*paraffins do not exhibit such a regular pattern. For example, with an *iso*paraffin the regular pattern is interrupted by a peak of very large intensity at the mass corresponding to the formation of the alkyl ion by rupture of the side chain.

In addition to the major peaks found at the masses calculated from the molecular formulae, both parent and

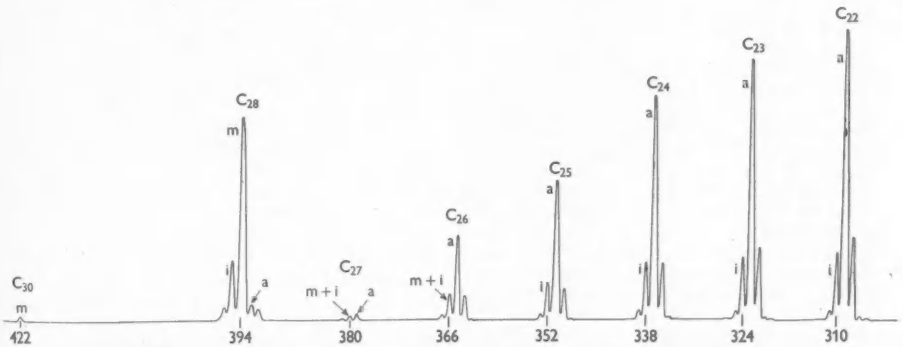

Fig. 2. Partial mass spectrum of the normal paraffin derived from wheat. For further explanation see text.

fragment ions give small isotope peaks (designated 'i' in Figs. 2 and 3). These are produced by ions in which one or more carbon atoms is substituted by the heavy isotope, ^{13}C, or in which a hydrogen atom is replaced by deuterium. For example, the peak at mass 395 in Fig. 2 is due to the molecular ions $^{12}C_{27}{}^{13}CH_{57}{}^{+}$ and $^{12}C_{28}H_{56}D^{+}$ and the peak at mass 338 is not due to the molecular ion $C_{24}H_{50}{}^{+}$ but to the alkyl ions $^{12}C_{23}{}^{13}CH_{49}{}^{+}$ and $^{12}C_{24}H_{48}D^{+}$. The intensity of the isotope peaks can be calculated exactly from the intensity of the unsubstituted ion peak and the known isotopic abundance of ^{13}C and deuterium. The method of calculation is discussed by Washburn (1950).

Apart from isotopic ions, for which correction can be made, a *n*-paraffin does not produce on electron bombardment any ions at mass numbers corresponding to the molecular weights of other *n*-paraffins. This forms the basis of the mass-spectrometric method of analysis. Thus in Fig. 2 it can be seen that the paraffin derived from wheat is mainly *n*-C_{28} with a trace of *n*-C_{30}, and, after correction for the isotopic contributions, traces of *n*-C_{27} and *n*-C_{26}. Similarly, in Fig. 3 the mass spectrum of the paraffin derived from runner bean indicates that this is mainly *n*-C_{28}, but that substantial amounts of other *n*-paraffins, in particular *n*-C_{26}, are also present.

In detail, the method of analysis consists of five steps: (1) At least five repeat scans of the mass spectrum are recorded. (2) The average intensity of the ion current at the molecular-ion peaks, $C_nH_{2n+2}{}^{+}$, is tabulated at each carbon atom number. (3) The contribution of the isotope of the alkyl ion, $C_nH_{2n+1}{}^{+}$, at the $C_nH_{2n+2}{}^{+}$ peak is calculated and subtracted from the measured value. (4) The value remaining is then due entirely to the molecular ion and this is divided by the appropriate relative sensitivity. (5) The quotients are summed, normalized to 100 % and the individual values reported as mole/100 moles concentration.

The relative sensitivity in step (4) is simply the intensity of the molecular-ion peak/unit pressure; the value varies with the molecular weight of the paraffin and is established by calibration. In the present work the instrument was calibrated with synthetic mixtures made with the paraffins listed in Table 2 and some *n*-hexatriacontane obtained from the American Petroleum Institute. In Table 1 the values for paraffins C_{22}, C_{26}, C_{27}, C_{28}, C_{29} and C_{36} were determined experimentally. A smooth curve of relative sensitivity against carbon atom number was then plotted, and from this values for the relative sensitivities of the other paraffins were obtained by interpolation.

Accuracy and limits of detection

The lack of pure normal paraffins of each carbon atom number for calibration means that the relative sensitivities used may be in error, possibly by 2–3 %. The method of analysis is such that an error in relative sensitivity is reflected directly as an error in concentration, and the absolute values given in Tables 2–5 therefore may be in error by 2–3 % of the reported concentration. Thus, for example, in Table 4 the values for C_{22} and C_{23} paraffins in fraction 12 could be in error by as much as 1·0 mole/100 moles, but the smaller concentrations such as those for C_{21} and C_{24} will be relatively unaffected. In the last-named the error is likely to be no more than 0·1 mole/100 moles. From the point of view of the present work the accuracy of the calibration data is relatively unimportant since the main interest is in the presence or absence of small concen-

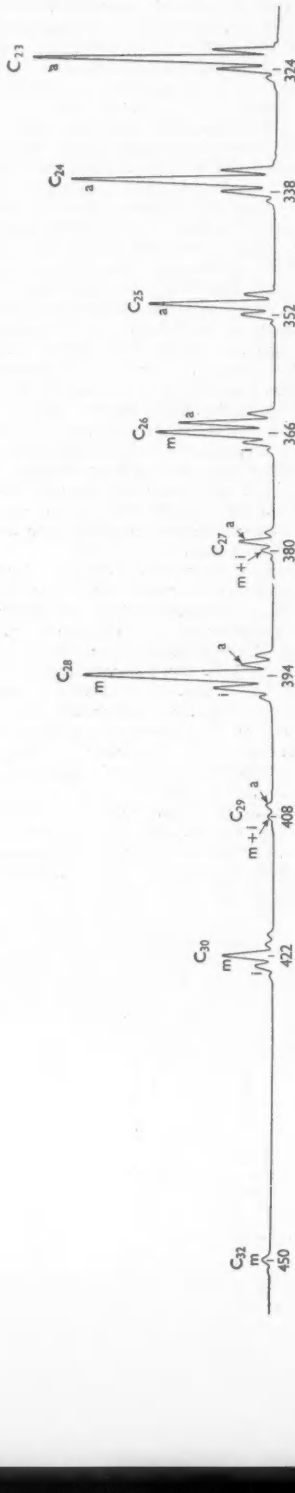

Fig. 3. Partial mass spectrum of the normal paraffin derived from runner bean. For further explanation see text.

Table 1. *Relative sensitivity of normal paraffin hydrocarbons*

C no.	21	22	23	24	25	26	27	28	29
Rel. sensitivity	4·3	3·5	2·7	2·1	1·55	1·3	1·15	1·00	0·88
C no.	30	31	32	33	34	35	36	37	38
Rel. sensitivity	0·79	0·73	0·67	0·64	0·60	0·58	0·56	0·54	0·52

trations of odd homologues in mixtures containing the even-number members of the series and vice versa. It is, however, important to consider the factors which affect the limit of detection. These are: (1) The minimum ion current that can be detected. (2) The accuracy of measurement of small peaks. (3) The accuracy with which the isotopic correction can be determined (step 3 in the above-described calculation).

Compared with the first two, factor (3) can be ignored since isotope ratios are known to be better than 0·5%. The limit of detection of higher paraffins in mixtures of lower paraffins is determined primarily by the first factor and the limit of detection of lower paraffins in mixtures of higher paraffins is determined primarily by the second. In both cases the predominant instrumental effect is the noise level of the system. With the instrument used the signal–noise ratio of the molecular-ion peak with a pure sample of *n*-octacosane was approx. 300:1. The limit of detection of *n*-octacosane is thus 0·3% and figures for other paraffins can be readily calculated from a knowledge of the appropriate relative sensitivity.

RESULTS AND DISCUSSION

The mass-spectrograph analyses of the synthetic *n*-paraffins (Table 2) show, for example, that the sample of lauric acid used to prepared C_{23} had contained about 2% of myristic acid, and that the samples of myristic acid used for C_{26}, C_{27} and C_{28} had contained about 1% of lauric acid and 2% of palmitic acid together with (in one case) about 1% of *n*-tridecanoic acid. These preparations had been made at a time when the criteria necessary to define the purity of a *n*-fatty acid were only slowly emerging, from the pioneer work of Francis, Piper & Malkin (1930) and of Malkin (1930), and when the application of X-ray analysis to long-chain compounds had not yet been fully exploited. Under the circumstances it is gratifying to find that these early synthetic paraffin preparations were as pure as the new analyses indicate.

The paraffins isolated from plant sources (Table 3) fall into two groups. In the first of these the products had been submitted to a laborious fractional crystallization with the object of identifying if possible the minor as well as the major components present. Apple-cuticle wax was an example. This contained 85–90% of C_{29} paraffin, and an examination of the residual fractions for shorter-chain material by comparing the melting-point and long-crystal-spacing data with those of appropriate synthetic mixtures of C_{29}–C_{28} and C_{29}–C_{27} paraffin led to the conclusion that the minor component was C_{27} and not C_{28}. The issue, unfortunately, could have been fogged at the time by the presence of about 0·5% of C_{28} in the synthetic C_{29} standard (Table 2), an amount as large as that now suggested as a minor component in the purified apple product (C134). For a similar reason the small percentage of even-number paraffins in Brussels-sprout wax would have been missed. The other group of plant paraffins had been given a more cursory examination. A fairly simple fractionation had shown that in each case the product contained more than one component, and by comparison with appropriate synthetic mixtures a satisfactory resolution in terms of the odd-number members seemed the most probable. It is clear, however, from the results given in Table 3, that although the odd-number members do indeed largely predominate, in no case was the interpretation on such an exclusive basis valid. All the even-number members between C_{28} and C_{34} are present as minor components in one or other of these waxes. The new finding receives confirmation from the revision of the rose-petal data (Table 4). The fractions that have been re-examined represent but a small proportion of the original paraffin mixture and each one was known to be of complex composition. In agreement with Ivanoff *et al.* (1955) the results show that all the even-number members from C_{22} to C_{30} are represented in one or other of these particular fractions. Unfortunately a sample of the original paraffin mixture was no longer available for overall quantitative analysis, and in the present research no more can be claimed than that the even-number homologues were minor components. The mass spectra of fractions 58 and 77 indicate that small quantities of *iso*paraffins were present. Apart from green tobacco leaf, which is discussed below, these were the only cases in which any *iso*paraffin was detected.

Carruthers & Johnstone (1959) have recently prepared hydrocarbon fractions from both green and fermented tobacco leaves and have had these submitted to mass-spectrographic analysis at the Thornton Research Centre, Shell Research Ltd. In each case the main component *n*-paraffin present was C_{31}, but appreciable quantities of the neighbouring odd-number homologues and small amounts of the even-number homologues were also present. The observed peak intensities, moreover, suggested that about half of each fraction consisted of *iso*-paraffins of chain-length similar to those just

Fig. 3. Partial mass spectrum of the normal paraffin derived from runner bean. For further explanation see text.

Table 2. *Analysis of synthetic paraffins*

Paraffin	Series no.	Composition (moles/100 moles) No. of C atoms 21	22	23	24	25	26	27	28	29	30	31	32	33
$C_{23}H_{48}$	B 491	—	—	98·4	—	1·6	—	—	—	—	—	—	—	—
$C_{26}H_{54}$	C 67	—	—	—	0·4	1·0	97·5	—	1·1	—	—	—	—	—
$C_{27}H_{56}$	B 13	—	—	—	—	0·8	—	97·3	—	1·9	—	—	—	—
$C_{28}H_{58}$	B 10	—	—	—	—	—	0·6	—	98·8	—	0·6	—	—	—
$C_{29}H_{60}$	C 141	—	—	—	—	—	—	1·4	0·5	97·3	—	0·8	—	—
Limit of detection		0·1	0·1	0·1	0·2	0·2	0·3	0·3	0·3	0·3	0·4	0·5	0·5	0·5

Table 3. *Analysis of paraffins isolated from plant sources*

Source	Analysis by mass spectrometry (M) or as mentioned in earlier work (E)	Composition (moles/100 moles) No. of C atoms 26	27	28	29	30	31	32	33	34	35	36	37	38
Apple peel (C134)	M	—	2·2	0·3	97·5	—	—	—	—	—	—	—	—	—
	E	—	—	—	100	—	—	—	—	—	—	—	—	—
Apple peel (C136)	M	—	12	1·8	86·2	—	—	—	—	—	—	—	—	—
	E	—	17	—	83	—	—	—	—	—	—	—	—	—
Swede-turnip leaf	M	—	1·3	1·3	95·0	—	2·3	—	—	—	—	—	—	—
	E	—	—	—	95	—	5	—	—	—	—	—	—	—
Brussels sprout	M	—	0·4	0·5	87·6	1·7	9·8	—	—	—	—	—	—	—
	E	—	—	—	(approx.) (90)	—	2·5–10	—	—	—	—	—	—	—
Cocksfoot grass	M	—	1·3	1·5	35·2	3·5	48·8	—	9·7	—	—	—	—	—
Runner-bean leaf	M	—	3·3	3	44	3·6	48	2·7	5·4	—	—	—	—	—
White-mustard leaf	M	—	0·7	0·8	35·7	3·7	52·4	3·2	3·5	—	—	—	—	—
	E	—	—	—	35	—	65	—	—	—	—	—	—	—
Rye grass	M	—	6·5	1·2	40·2	4·6	39·2	—	8·3	—	—	—	—	—
	E	—	—	—	Too complex to suggest			—	—	—	—	—	—	—
Green tobacco leaf*	M	—	—	—	~5	~5	~60	~10	~20	—	—	—	—	—
	E	—	—	—	†	—	†	—	†	—	—	—	—	—
Cactus leaf	M	—	—	—	—	—	2·6	1·5	14·7	5·8	64·6	—	10·8	—
	E	—	—	—	—	—	—	—	†	—	†	—	†	—
Limit of detection		0·3	0·3	0·3	0·3	0·4	0·5	0·5	0·5	0·5	0·6	0·6	0·6	0·6

* Contains substantial quantities of *iso*paraffin; values must therefore be regarded as approximate.

† Detected.

Table 4. *Analysis of rose-petal paraffin fractions*

* Contains substantial quantities of *iso*paraffin; values must therefore be regarded as approximate. † Detected.

Table 4. *Analysis of rose-petal paraffin fractions*

	Composition (moles/100 moles)										
	No. of C atoms										
Fraction no.	21	22	23	24	25	26	27	28	29	30	31
12	4·1	42·6	48·7	4·6	—	—	—	—	—	—	—
27	—	1·6	29·7	33·7	26·1	6·2	2·7	—	—	—	—
58*	—	—	—	—	—	1	2	21	47	3	2
77*	—	—	—	—	—	1	1	2	40	24	32
Limit of detection	0·1	0·1	0·1	0·2	0·2	0·3	0·3	0·3	0·3	0·4	0·5

* Mass spectra suggest that these samples may also contain small quantities of *iso*paraffin.

Table 5. *Analysis of paraffins derived from various primary alcohols*

	Analysis by mass spectrometry (M) or as mentioned in earlier work	Composition (moles/100 moles)										
		No. of C atoms										
Source	(E)	24	25	26	27	28	29	30	31	32	33	34
Barley leaf	M	1·1	—	94·4	—	4·5	—	—	—	—	—	—
Cocksfoot grass	M	—	—	94·6	—	5·4	—	—	—	—	—	—
Wheat blade	M	—	—	0·5	0·3	98·5	—	0·7	—	—	—	—
	E	—	—	—	—	100	—	—	—	—	—	—
Apple peel	M	—	—	5·8	1·7	66·8	4·8	20·9	—	—	—	—
	E	—	—	*	—	*	—	*	—	—	—	—
Wild-white-clover leaf	M	—	—	10·8	1·8	26·1	2·0	59·3	—	—	—	—
Runner-bean leaf	M	—	—	16·1	0·3	50·8	0·8	24·1	0·6	7·3	—	—
Cactus leaf	M	—	—	1·1	1·2	52·2	6·8	31·5	2·3	4·9	—	—
	E	—	—	—	—	50	—	50	—	—	—	—
Lucerne leaf	M	—	—	—	—	2·2	2·2	95·1	—	0·5	—	—
	E	—	—	—	—	—	—	99	—	—	—	—
White-mustard leaf	M	—	—	—	—	15·2	2·7	35·2	4·2	29·2	—	13·6
	E	—	—	—	—	20	—	40	—	40	—	—
Lac wax	M	—	—	—	—	27	0·6	20·7	0·5	33	—	18·3
	E	—	—	*	—	*	—	*	—	*	—	*
Limit of detection		0·2	0·2	0·3	0·3	0·3	0·3	0·4	0·5	0·5	0·5	0·5

* Detected.

mentioned. The corresponding fractions prepared by Chibnall *et al.* (1934), which were thought at the time to consist largely of C_{31} with subordinate amounts of C_{29} and C_{33} paraffins have now been passed through the mass spectrometer. In agreement with Carruthers & Johnstone (1959) the new analyses confirmed the presence of small amounts of the even-number homologues and also of *iso*-paraffins (Table 3). It is clear from all these new findings that the older methods of fractionation employed by Chibnall and his colleagues were not sufficiently selective to resolve binary and ternary mixtures containing about equal amounts of neighbouring homologues and that the composition of such mixtures could not be predicted with certainty from melting-point and long-crystal-spacing data.

A similar story emerges from the re-examination of the paraffins obtained by reduction via the iodide from the wax primary alcohols (Table 5). The new analysis confirms the earlier deduction that the paraffin from the wheat primary alcohol was as pure a sample of C_{28} as the synthetic standard used for comparison (Table 2). The lac-wax primary alcohols had been extensively fractionated and in this case the new analysis of the bulked material confirms the presence of the even-number members from C_{28} to C_{34}, the odd-number members being virtually absent. In all the other cases, however, one or more of the odd-number members are definitely present as minor components. From the known relationship between the *n*-fatty acids and primary-alcohol constituents of waxes it may be inferred that the odd-number acids may also be minor components in some cases.

SUMMARY

1. A number of the *n*-paraffin and primary-alcohol fractions of plant waxes characterized in former years by Chibnall and his colleagues have been re-examined by mass spectroscopy.

2. Contrary to the earlier suggestion that the *n*-paraffin fractions comprised exclusively the odd-number members of the series, it is now shown that in some cases the even-number members may be present as minor components.

3. Likewise the primary-alcohol fractions are not composed exclusively of the even-number members of the series; in some cases the odd-number members may be present as minor components.

4. *iso*Paraffins are present in rose-petal wax and tobacco-leaf wax but in no other wax examined in the present research.

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The Assay of Human Plasminogen with Casein as Substrate

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Casein has been widely used for the assay of proteolytic enzymes since Kunitz (1947) described his procedure for the measurement of the activity of trypsin and chymotrypsin. Several procedures have since been designed for the assay of plasmin, the most widely used being that of Remmert & Cohen (1949). These workers activated plasminogen with excess of streptokinase in the presence of casein and then casein was added to the reaction mixture. After incubation the protein was precipitated and the tyrosine content of the filtrates estimated. A proteolytic unit was defined as the amount of enzyme producing an increase of 450 μg. of acid-soluble tyrosine in an hour at 35° in a medium containing 4 g. of casein/100 ml.

For work on the purification of human plasminogen it was intended to use an assay based on that described by Müllertz (1955), who purified casein and measured hydrolysis by the increase in extinction at 275 mμ after precipitating protein with perchloric acid.

With our procedure, when a balance sheet was made of the activities of fractions obtained during the course of purification experiments, the total activity exceeded that of the starting material by 12–20%. Moreover, for any particular preparation, the estimated activity varied with the time allowed for the interaction of the enzyme with the substrate: the longer the incubation the higher the resulting activity. By examining samples taken at short intervals from the incubation mixture, the course of the reaction was found to be triphasic: an initial non-linear region of increasing slope, then a linear region followed by a region of decreasing slope. From this it was clear that an assay based on a single sample taken at a fixed time from the initiation of the reaction would be unreliable because of the initial non-linear phase. Many experiments were made under different conditions, with several plasmin preparations. It was found that a closer agreement between the total activity of the fractions and that of the starting material was obtained when the slope of the linear part of the curve was used for calculating the activity. The present work was undertaken to establish the validity of an assay based on this calculation.

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MATERIALS AND METHODS

Buffers. Phosphate buffers (I 0·2) containing Na^+ and K^+ ions and covering the range pH 7·0–8·0 were prepared according to Sørensen (1909). Phosphate–NaCl: 0·19 M-NaCl (900 ml.), phosphate buffer, pH 7·6, 100 ml. Acetate–phosphate buffers, pH 4–6 (Kekwick & Mackay, 1954).

Plasminogen. Plasminogen was prepared from out-dated citric acid–sodium citrate–dextrose plasma as described by Derechin (1960) for the fraction BL. After precipitating fibrinogen from 2 l. of plasma the supernatant solution was adjusted to pH 5·3 with 0·5 M-acetic acid, the volume made up to 4 l. with water and ether was added to a concentration of 10% (v/v) at 0°. The resulting precipitate, which contained prothrombin and plasminogen, was redissolved in 150 ml. of 0·049 M-trisodium citrate; the volume was made up to 750 ml. with water and ether was added to a concentration of 10% (v/v) at −3°. The precipitate was redissolved in 1 l. of acetate–phosphate buffer, pH 4·0; the pH of the solution was adjusted to 5·15 with acetate–phosphate buffer, pH 6·0, and the volume made up to 6 l. with water at 0°. The precipitate was dissolved in 1 l. of phosphate–NaCl and portions (100 ml.) of this solution were shaken with 200 ml. of ether at −3° in transfusion bottles (540 ml.). The precipitate was removed by centrifuging and plasminogen was precipitated from the bottom solution by dialysis for 36 hr. against three changes of 4 l. of acetate–phosphate buffer, pH 5·15.

Streptokinase. A commercial preparation (Varidase, Lederle) was used. The activity (Christensen, 1949) was taken to be that indicated on the vials. Fresh solutions were prepared daily in phosphate buffer, pH 7·6.

Plasmin. Plasminogen solutions were mixed with optimum amounts of streptokinase, in the proportion 4:1 (v/v) respectively, and were allowed to stand for 10 min. at room temperature (18–20°) to complete the activation.

Casein. Purified casein was prepared by a method based on the procedure of Müllertz (1955). Commercial casein (20 g.; Hammersten, British Drug Houses Ltd.) was dissolved in water (600 ml.) by adding 0·2 N-NaOH slowly so that at no stage did the pH exceed 8. The solution was boiled and after cooling (2–4°) was brought to about pH 2·0 with N-HCl. At this temperature the precipitate that formed near the isoelectric point was much finer and redissolved more easily on decreasing the pH, than that at room temperature. The solution was then treated with 900 ml. of 0·17 M-perchloric acid and the resulting precipitate was washed four times with water (1 l.), suspended in

phosphate buffer, pH 7·6 (200 ml.), and dialysed at 2–4° for 3 days against six changes of the same buffer. The precipitate dissolved completely overnight. This method provided a medium of constant ionic composition and removed undesirable perchlorate ions from the washed precipitate. After the dialysis the protein concentration of the solution was measured with a dipping refractometer and adjusted to the required concentration. It was then filtered through Seitz EK filters to give a clear solution. The yield of casein was 50–60%.

Solutions of commercial casein were prepared by suspending it in phosphate buffer, pH 7·6, and dialysing against the same buffer at 2–4° as already described. Some of these solutions were boiled. Occasionally commercial casein solution was prepared by dissolving in phosphate buffer, pH 7·6, and readjusting the pH to 7·6 with 0·2 N-NaOH.

Assay procedure. Plasmin was added to a casein solution at 40° in the proportion 1:3 (v/v). At convenient intervals samples (4 ml.) of this reaction mixture were withdrawn and pipetted into 2 ml. of 1·7 M-perchloric acid at room temperature and shaken at intervals for 20–30 min. until most of the precipitated protein fell spontaneously to the bottom of the tube. The suspension was filtered and the extinction of the filtrate was read at 276 mμ in 1 cm. cells with a Unicam model SP. 600 spectrophotometer. Readings were made for each mixture against a blank prepared by adding 1 ml. of plasmin and 3 ml. of casein solution to 2 ml. of 1·7 M-perchloric acid. For establishing the reaction curves, times were measured from the moment of adding the plasmin to casein. The protein precipitated from the samples by perchloric acid could be removed by centrifuging or filtering. Centrifuging did not give particle-free material and solutions were therefore filtered through washed Whatman no. 1 paper. Unwashed paper introduced material with apparent absorption at 276 mμ. This effect was considerable when Whatman no. 3 or no. 42 was used.

Unless otherwise stated the conditions of the tests were: temperature 40°; pH 7·6; I 0·2; casein concentration 2·25 g./100 ml. in the reaction mixture.

RESULTS

Conditions for the activation of plasminogen to plasmin

Protection of plasmin by the casein in the activation mixture. Remmert & Cohen (1949) activated plasminogen with streptokinase for 10 min. in a medium containing 1·35 g. of casein/100 ml., at 35°. No reason was given for the presence of the substrate in the activation mixture. Kline (1954) observed that casein and other substrates prevent the inactivation of the plasmin formed by streptokinase activation of plasminogen. This effect of casein was studied at several temperatures. The results agreed with Kline's (1954) conclusions when activation was carried out above 30°. In these circumstances a higher plasmin activity was obtained in the presence of casein. However, when the activation took place at or below 30° the plasmin activity obtained was uninfluenced by the presence of casein, and reached the maximum value.

Incubation time for full activation. Plasminogen samples were activated with an optimum concentration of streptokinase at room temperature (18–20°) for 10, 30 and 60 min. respectively before being added to casein (Fig. 1). To obtain results for zero activation time plasminogen and streptokinase were added separately to casein. The linear part of the reaction curves obtained had the same slope in all instances, but with zero activation time the linear phase was 2 min. delayed with respect to the curve given by the other three samples. This suggests that under the experimental conditions used, 2 min. is required for maximum activation to occur.

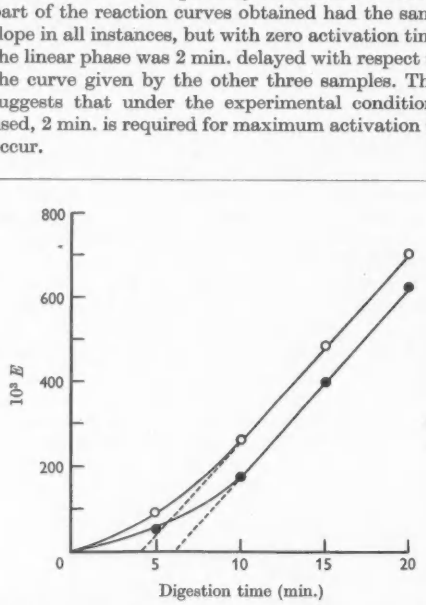

Fig. 1. Time required for maximum activation of streptokinase–plasminogen mixtures. Streptokinase in the activation mixture: 200 units/ml. Plasminogen in the reaction mixture: 0·15 mg./ml. ○, 10, 30 and 60 min.; ●, 0 min. The interval between the intercepts of the extrapolations to the abscissa indicates the minimum time for maximum activation.

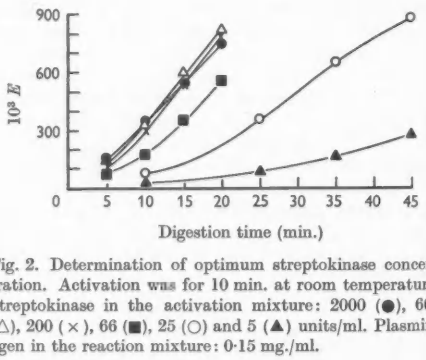

Fig. 2. Determination of optimum streptokinase concentration. Activation was for 10 min. at room temperature. Streptokinase in the activation mixture: 2000 (●), 660 (△), 200 (×), 66 (■), 25 (○) and 5 (▲) units/ml. Plasminogen in the reaction mixture: 0·15 mg./ml.

Determination of optimum concentration of streptokinase. With a constant activation time of 10 min. at room temperature, the streptokinase concentration of the activation mixture was varied from 5 to 2000 units (Fig. 2).

The slope of the curves increased with time, and with streptokinase concentration up to 200 units/ml. At 200 units/ml. the slope was clearly linear and remained constant for mixtures containing 200–660 units/ml., falling from this constant value by 15% at 2000 units/ml. There was thus a broad optimum range of streptokinase concentration between 200 and 660 units/ml. in the activation mixture, in which maximum activation occurred with an activation time of 10 min.

In a further experiment a suboptimum concentration of streptokinase (25 units/ml.) was tested for periods of 10–200 min. A control containing the optimum concentration of streptokinase (200 units/ml.) was activated for 10 min. Provided that sufficient time was allowed, plasminogen was maximally activated at a streptokinase concentration of 25 units/ml.

Optimum temperature and pH *for the proteolysis of casein*

A study of the effect of temperature on the reaction curves showed that the steepest linear slope occurred at 45° (Fig. 3*A*). However, at 40° the concentration of the products of reaction rose to a higher value than at 45° before the linear phase of the reaction ended. This suggested that at 45° there was a significant loss in enzyme activity during the course of the measurements. There was more obvious inactivation of the enzyme at 50°.

In Fig. 3*B* it can be seen also that the activity at 40° was about 2·5 times that at 30° and that in this region the activity increased linearly with temperature.

An examination of the effect of pH on the activity showed that the optimum occurred between 7·4 and 7·6, which is in agreement with the results of other workers (Christensen & MacLeod, 1945; Remmert & Cohen, 1949).

The standard conditions selected for the assay of plasmin with casein accordingly were pH 7·6, temperature 40°.

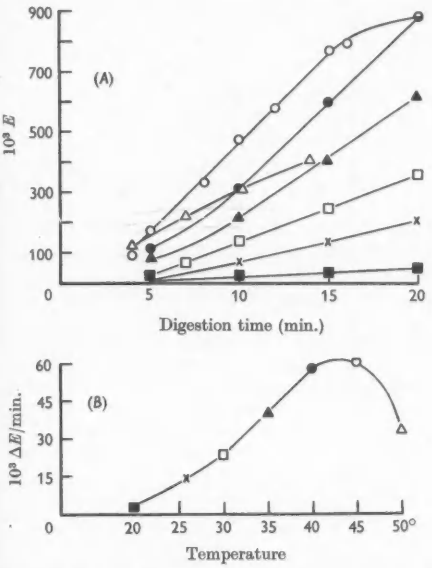

Fig. 3. Influence of temperature on enzyme activity. Streptokinase in the activation mixture: 200 units/ml. Plasminogen: 0·17 mg./ml. in the reaction mixture. (*A*) Reaction curves at 20° (■), 26° (×), 30° (□), 35° (▲), 40° (●), 45° (○) and 50° (△). (*B*) Rate of enzyme action plotted against temperature. Calculations were made from the slope of the linear part of the reaction curves of Fig. 3*A*.

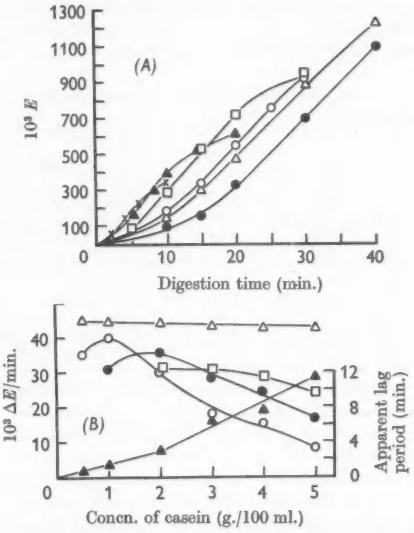

Fig. 4. Influence of casein concentration on the proteolysis by plasmin. Streptokinase: 200 units/ml. in the activation mixture; plasminogen: 0·15 mg./ml. in the reaction mixture. (*A*) Reaction curves with 0·5 (×), 1·0 (▲), 2·0 (□), 3·0 (○), 4·0 (△) and 5·0 (●) g. of casein/100 ml. of the reaction mixture. (*B*) Rates of enzyme action calculated from the slope of the linear part of the curves (△) and from single determinations after proteolysis for 10 (○), 20 (●) and 30 (□) min. The apparent lag period (▲) was obtained by extrapolation of the linear part of each curve to the abscissa.

Effect of substrate concentration

Six casein concentrations varying from 0·5 to 5 g./100 ml. were tested (Fig. 4*A*). The higher the substrate concentration the longer the lag period before the linear part of the curve was reached. On the other hand, the lower the substrate concentration the shorter the linear phase of the reaction, though the slope of the linear part was very little affected. A tenfold increase in substrate concentration resulted only in a 7 % decrease in the slope of the linear section of the curve. However, when the rate of the reaction was calculated on the basis of a single reading, a fivefold increase in casein concentration produced apparent differences of the order of 25–80 % in the activity of the same enzyme preparation.

It was clear that the substrate concentration was not critical for the calculation of activity from the slope of the linear part of the reaction curve. A final concentration of about 2 g. of casein/100 ml. was chosen. With it the lag period was not too long and allowed sufficient time for the assay, provided that the plasminogen concentration was adjusted accordingly. Lower casein concentrations were occasionally found useful for assaying weak plasminogen preparations. In no instance was a casein concentration less than 1 g./100 ml. used.

Effect of plasminogen concentration

When the plasminogen concentration was increased at constant casein concentration, the slope of the linear portion of the reaction curve increased proportionally and the lag period was progressively shortened. When the rate calculations were made from a single reading at 20 min., the rate–enzyme concentration curve was convex to the abscissa (Fig. 5).

Results with commercial preparations of casein

The behaviour of commercial casein, undialysed and dialysed for 3 days against phosphate buffer, was compared with that of purified casein. In several preparations of commercial casein, and of purified casein incubated at 40° for 2 hr., there was no increase in ultraviolet-absorbing material soluble in perchloric acid in the absence of plasmin. A certain amount of perchloric acid-soluble, ultraviolet-absorbing material present in the undialysed commercial preparations could be completely removed by dialysis. With different batches of casein there were slight variations in the rate of hydrolysis up to 15 %, when the same enzyme preparation was assayed. The results suggested that the purification procedure of Müllertz (1955) had little effect on the accuracy of the assay: about 40–50 % of the casein was lost during the purification.

Standard assay conditions

It was clear that the determination of the slope of the linear part of the reaction curve was the most reliable basis for the assay of plasmin with casein. The reagents are handled as described in the Materials and Methods section. In addition, when room temperature was higher than 30° a casein solution (3 g./100 ml.) was used as the solvent for streptokinase instead of phosphate buffer.

To determine the slope of the linear part of the reaction curve two procedures were adopted. When the activity of the enzyme preparation was unknown a complete curve was constructed, comprising not less than six points covering a period of 30 min. from the addition of plasmin to the substrate. A blank of water was then used in determining the extinction of the filtrates. When the order of activity of the enzyme preparation was known, the amount of enzyme in the reaction mixture was adjusted to ensure that three samples taken at intervals of 3–5 min. from one another would each give estimates falling on the linear portion of the reaction curve. In all, four samples were taken, the

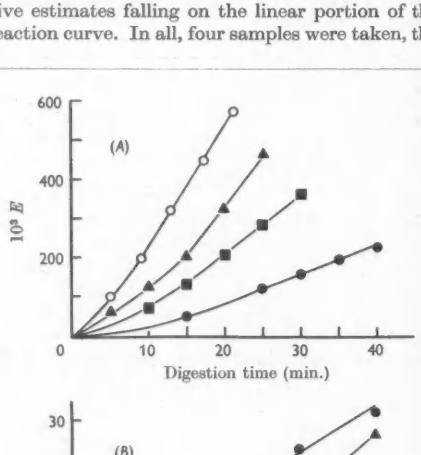

Fig. 5. Relation between enzyme concentration and activity. Streptokinase: 200 units/ml. in the reaction mixture; plasminogen: 1·0 = 0·10 mg./ml. in the reaction mixture. (*A*) Reaction curves with four concentrations of plasmin: 1·0 (○), 0·75 (▲), 0·5 (■) and 0·25 (●). (*B*) Rates of enzyme activity calculated from the linear portion of the curves (●) and from a single determination after digestion for 20 min. (▲).

first, after proteolysis for 10 min., being used as a blank.

The plasmin unit defined here is that amount of enzyme which gives rise to an increase of 1×10^{-3} unit of extinction/min., measured at 276 mμ during the linear phase of the reaction with a casein concentration of about 2 g./100 ml. in phosphate buffer, pH 7·6 (I 0·2), at 40°.

The specific activity of a preparation is given by the equation: specific activity $= 10^3(SF)/N$, where S = slope of the linear portion of the reaction curve in units of extinction/min., F = the overall dilution factor, N = mg. of N/ml. of the plasminogen solution assayed.

The overall dilution factor is the product of three terms: the dilution of the plasminogen solution with streptokinase at the activation stage, the dilution when the activated product is added to the casein substrate and the dilution when samples of the digestion mixture are added to perchloric acid. In the standard test these are 1·25, 4·0 and 1·5 respectively, leading to an overall factor of 7·5.

The use of suitable dilution factors also makes possible comparison between assays performed with different proportions of the reagents.

DISCUSSION

The reaction curve of the plasmin–casein interaction in these experiments is triphasic whatever the experimental conditions. This result is not due to incomplete activation of the enzyme. The presence of an inhibitor in the enzyme preparation would be expected to give a curve concave to the abscissa when rate of reaction is plotted against enzyme concentration. This did not occur (Fig. 5B) in curves based on estimates either by the linear-slope method or from a single reading; with the linear-slope method the enzyme activity is directly proportional to its concentration. The plots of the curves showing the effect of the substrate concentration based on a single reading after a set period (Fig. 4B) might suggest the presence of an inhibitor in the casein preparations. However, the slope of the linear part of the reaction curves is very little modified by a tenfold increase in substrate concentration and the reaction proceeds at its maximum rate for a longer period the higher the substrate concentration.

A similar type of reaction curve was obtained for the hydrolysis of redistilled ethyl mandelate by liver aliesterase by Willstätter, Kuhn, Lind & Memmen (1927). In this case, an increase in the substrate concentration by a factor of two approximately doubled the lag period. This was due to the presence in the substrate solution of small amounts of its keto ester, which had a strong affinity for the enzyme but was hydrolysed at a much slower rate. The removal of the keto ester allowed the reaction to proceed linearly from the starting point.

The observation of this 'anomalous' behaviour of casein when used as a substrate for proteolysis is not new. A lag period can be seen very clearly in the reaction curves published by Remmert & Cohen (1949); but in spite of this they based their assay procedure on a single reading after digestion for 60 min. Christensen (1954) also obtained a non-linear curve when he studied the action of trypsin, chymotrypsin and plasmin on casein. Müllertz (1955) on the contrary obtained no lag period; his curves are linear to the origin. The low casein concentration in the digestion mixture may explain this result. Although Müllertz designates a stock casein solution containing 3 g./100 ml. the actual concentration is likely to have been below 2 g./100 ml. because of losses occurring during purification. The casein concentration of the reaction mixture would consequently be below 1 g./100 ml. and at this concentration the lag period is rather short. Further, he measured the absorption for zero time from samples taken after adding the plasmin to casein. It is unlikely that such samples could be handled in much less than a minute and this procedure would also shorten the apparent lag period.

The type of reaction curve described here might be due to a complex sequence of interactions between plasmin and casein. Bergman (1938) has shown that the action of a proteolytic enzyme on a substrate is affected by a number of variables such as the length of the peptide chain, the ratio and sequence of the individual amino acid residues and the nature of the side chains. Changes in the peptide chains with the progress of the hydrolysis might determine a change in the rate of the reaction. A peptide bond hydrolysed at a low rate owing to the presence of certain groups in the neighbourhood might be hydrolysed at a higher rate after the removal of these groups. Also, the shortening of the peptide chain might have a similar effect.

The fact that casein is not a homogeneous protein makes the situation more complicated. Mellander (1939) showed that casein is composed of at least three electrophoretic components, which he designated as α, β and γ, in the order of decreasing mobilities at alkaline pH values. The triphasic-reaction curve obtained when casein is digested with plasmin might be explicable in part on the basis of a differential rate of hydrolysis of these components, an explanation similar to that given by Willstätter *et al.* (1927) for the hydrolysis of ethyl mandelate. There is no evidence at present available to support this suggestion.

Experimental work on the proteolysis with plasmin of separated casein components (Derechin, 1960) indicates that the protein composition of casein mixtures is a controlling factor determining the shape and slope of the curves.

SUMMARY

1. When the proteolysis of casein by plasmin is followed by measuring the increase in material with an absorption at 276 mμ soluble in perchloric acid, a triphasic curve is obtained, the second part of which is linear.

2. The assay of plasminogen preparations from single determinations after a fixed time of proteolysis gives unreliable results because of the influence of the lag period before the linear portion of the curve is reached.

3. The length of the lag period before the linear portion of the reaction curve is reached is increased by increasing the substrate concentration or by decreasing the enzyme concentration.

4. The slope of the linear part of the curve is proportional to enzyme activity and is almost independent of substrate concentration over a tenfold range.

5. A standardized assay is described which gives reliable results, based on the slope of the linear part of the reaction curve.

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